MISMATCH REPAIR & HYPOXIA IN REPAIR OF CISPLATIN-DAMAGED DNA

THE EFFECTS OF MISMATCH REPAIR PROTEINS AND HYPOXIA ON THE REPAIR OF CISPLATIN-INDUCED DNA DAMAGE

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Abstract

Tumour resistance to the drug cisplatin poses a major impediment to successful chemotherapy. Defects in the mismatch repair (MMR) system have been linked to cisplatin resistance; however, conflicting reports have suggested that loss of MMR alone may not be sufficient to confer resistance in some cell types. Rapidly growing tumours often outstrip their blood supply resulting in a hypoxic tumour microenvironment which has been shown to affect cellular responses to conventional cancer treatments. However, the effect of hypoxia on the repair of cisplatin-damaged DNA has not been investigated. We have used a host cell reactivation (HCR) assay to investigate the effects of MMR deficiency, p53 defects, SV40-transformation and hypoxia (or hypoxia coupled with acidosis) on the repair of a cisplatin-damaged reporter gene encoded by a recombinant adenovirus in a panel of murine and human cells. We have also examined HCR of a UVC-damaged reporter gene in human normal cells for comparison and clonogenic survival of murine fibroblasts following cisplatin treatment. In the isogenic murine fibroblasts we examined, loss of the MMR proteins MSH2, MSH6 or PMS2 alone were insufficient to enhance HCR of the cisplatin-damaged reporter gene. However, HCR was enhanced in SV40-transformed MSH2-deficient cells relative to proficient controls. In the human tumour cells we examined loss of hMLH1 alone, but not hMSH2, resulted in enhanced HCR of the reporter gene. These results indicate that loss of MMR alone may not be sufficient to confer cisplatin resistance in some cells, suggesting that other concomitant alterations may also be necessary. We examined p53 defects and SV40-transformation changes as possible candidate alterations required for a cisplatin resistant

phenotype in Li-Fraumeni syndrome cells and SV40-transformed human normal cells respectively. We found that SV40-transformation resulted in enhanced HCR of the cisplatin-damaged reporter gene relative to non-transformed cells, but a similar repair enhancement was not seen for mutant p53-expressing Li-Fraumeni syndrome cells relative to control cells expressing wildtype p53. A comparison of repair of UVC- and cisplatin-induced DNA damage showed that HCR of the UVC-damaged reporter gene, but not the cisplatin-damaged reporter gene was reduced in SV40-transformed cells suggesting a differential effect of SV40-transformation-induced abrogation of p53 and/or pRb on nucleotide excision repair and homologous recombination repair in cells. The effects of hypoxia on the repair of cisplatin-damaged DNA varied with cell type although reduced repair under hypoxic conditions was observed in both SV40-transformed human normal cells and in several human tumour cells when cells were examined at 40 h post-infection. However, differential effects of hypoxia on repair capacity were observed in some cell lines when the length of hypoxic treatment was shorter and cells were examined at an earlier time point indicating rate-dependent effects on the repair of cisplatin-damaged DNA in some cells. There were no detectable effects of hypoxia plus low pH treatment (24 h post-infection) on repair of either cisplatin-damaged or UVC-damaged DNA in the human normal cells we examined suggesting that a longer duration of hypoxia and low pH treatment may be necessary to detect repair differences. Finally, clonogenic survival of SV40-transformed MSH2-deficient and MSH2-proficient murine cells treated with cisplatin revealed a hypoxia-enhanced survival of the MSH2-deficient cells. This enhancement of survival coupled with a reduction in repair capacity

previously observed in these cells under hypoxic conditions provides a potential mechanism by which hypermutability may be established in hypoxic cells.

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List of Abbreviations

8-OxoG	7,8-dihydo-8-oxoguanine
α-MEM	alpha minimum essential media
β-gal	beta-galatosidase
Ad	adenovirus
ATP	adenosine triphosphate
BER	base excision repair
BRCA	breast cancer
Cisplatin	*cis*-diamminedichloroplatinum(II)
CPD	cyclobutane pyrimidine dimer
CPRG	chlorophenolred-β-D-galactopyranoside
CS	Cockayne's syndrome (subsequent letters indicate complementation group i.e. CSA-complementation group A)
D_{37}	dose that reduces the surviving fraction of cells to 0.37
DNA	deoxyribonucleic acid
E1	early region 1
E. coli	*Escherichia coli*
ERCC1	excision repair cross-complementing 1
ExoI	exonuclease I
FA	Fanconi's anemia
G1/S	gap 1/synthesis phase of the cell cycle
GGR	global genome repair
GSH	glutathione
HCMV	human cytomegalovirus
HCR	host cell reactivation
hHR23B	human homologue of Rad23 B
HIF-1	hypoxia-inducible factor 1
hMLH1	human Mut L homologue 1
hMSH2	human Mut S homologue 2
hMSH3	human Mut S homologue 3
hMSH6	human Mut S homologue 6
HNPCC	hereditary nonpolyposis colorectal cancer
hPMS2	human post-meiotic segregation 2

HRR	homologous recombination repair
IE	immediate early
lacZ	β-galactosidase gene
LFS	Li-Fraumeni syndrome
MB+VL	methylene blue + visible light
MCMV	murine cytomegalovirus
Mdm2	murine double minute 2
MEFs	murine embryonic fibroblasts
MLH1	mut L homologue 1
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger RNA
MSH2	mut S homologue 2
MSH6	mut S homologue 6
MT	metallothionein
mtDNA	mitochondrial DNA
MutH	mut H endonuclease
MutLα	heterodimer of MLH1 and PMS2
MutSα	heterodimer of MSH2 and MSH6
MutSβ	heterodimer of MSH2 and MSH3
NDF	normal diploid fibroblast
NER	nucleotide excision repair
p53	protein 53
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
Pfu	plaque forming units
PMS2	post-meiotic segregation 2
pRb	retinoblastoma protein
Pt-AG	cisplatin 1,2-d(ApG) intrastrand adduct
Pt-GG	cisplatin 1,2-d(GpG) intrastrand adduct
RAD51	eukaryotic homologue of the prokaryotic recA recombinase
RF-C	replication factor C
RNA	ribonucleic acid
RPA	replication protein A
SE	standard error

SV40	simian virus 40
TCR	transcription-coupled repair
TFII	transcription factor for RNA polymerase II
UV	ultraviolet
wt	wildtype
XP	xeroderma pigmentosum (subsequent letters indicate complementation group i.e. XPA-complementation group A)
XRCC	X-ray repair cross-complementing

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CHAPTER 1

Introduction

1.0 DNA Damage and Cancer

The phenomenon of cancer has been recognized for centuries, but it wasn't until the introduction of molecular biology and genetics in the twentieth century that researchers began to understand its underlying mechanisms (Chabner and Roberts 2005). It has become clear that cancer is a genetic disease and as our understanding of the human genome has evolved so too has our understanding of this disease.

High fidelity replication of genetic material in cells hinges on the stability of deoxyribonucleic acid (DNA), the molecular structure that contains the "blueprints" for our development and functioning (Friedberg et al. 1995). However, somatic cells are continuously faced with exogenous and endogenous challenges that modify the structure of DNA. These include exposure to carcinogens in the environment (such as ultraviolet radiation and chemicals), replication errors and spontaneous mutations in DNA (Friedberg et al. 1995, Lodish et al. 2004). Cells have evolved multiple mechanisms including DNA repair pathways to maintain genome integrity; nevertheless, some mutations go unchecked and accumulate in the genome over time often leading to cancer. Thus, cancer is inherently linked to old age. Mutations in the germline may also predispose individuals to cancer and several heritable syndromes closely linked to cancer onset have been identified.

The classical paradigm of carcinogenesis defines the process in three stages: initiation, promotion and progression (as reviewed in Moolgavkar and Luebeck 2003). In the initiation stage a cell accumulates one or more mutations in critical genes involved in genome stability or cell cycle control (i.e. DNA repair gene, proto-oncogene). During the

promotion stage the mutated cell expands clonally giving rise to other initiated cells. Proliferation of initiated cells allows for additional mutations to accumulate in proto-oncogenes and/or tumour suppressor genes leading to malignant transformation of one or more cells. Given the role of oncogenes in driving cell cycle progression and inhibiting apoptosis and the role of tumour suppressor genes in inhibiting cell cycle progression, promoting apoptosis and promoting repair, malignant transformation typically results in tumour invasion and metastasis (Griffiths et al. 2000).

While progress has been made in understanding some of the genetic and molecular processes involved in many types of cancer, it is clear that further study is needed given the complexity of carcinogenesis and the uniqueness of this process in different cell types. Ultimately, as our understanding of the genetic underpinnings of cancer increases, clinicians will be able to individualize therapies for various types of cancer resulting in more effective treatment and better prognosis for patients.

2.0 Historical Perspective on Cancer Treatment

Early methods of cancer treatment primarily involved the removal of tumours through surgery. However, the discovery of X-rays in the late nineteenth century opened the door to a novel method of treatment that would prove useful for some inoperable cancers. Soon after their discovery, X-rays were being used to treat many types of cancer with some success for skin cancer but comparatively little or no success for deeper tumours (Fletcher 1988). Notably, important concepts that would become standard for other kinds of therapy emerged from work with x-rays including, dose-response

relationships, tumour classes based on radiosensitivity and quantification of tumour responses to therapy using surviving fractions (Fletcher 1988). Today radiotherapy is still common practice as a curative or palliative treatment depending on the tumour type and stage of advancement. Advancements in radiation technology such as linear accelerators have enabled clinicians to more closely target tumours for treatment (reviewed in Thwaites and Tuohy 2006) and combining radiotherapy with other treatment strategies has been effective for some tumour types (Armstrong et al. 1990, Rave-Fränk et al. 2007, Zhang et al. 2007).

Early on in studies of radiation therapy, it became increasingly apparent that while irradiation was able to shrink some tumours, it was unable to control cancer metastasis. This led to the search for other therapies that could affect the body on a much broader scale. Chemotherapy offered a promising alternative. Ironically, the very compounds that were used in chemical warfare became the focus of early studies in the search for chemical compounds of therapeutic value. Nitrogen gas, a compound related to sulphur mustard gas (used in World War I) was used to treat a cancer patient in 1942 constituting the first demonstration of anti-tumour effects, albeit temporary, with a chemical compound (as reviewed in Chabner and Roberts 2005). In the years to follow several other drugs were tested for antitumour properties with some success, but this was tempered by the discovery of acquired chemoresistance in some tumours and toxic side effects in patients. Among the success stories, was the discovery of cis-diamminedichloroplatinum(II) (also known as cisplatin), which was introduced into clinical use in the 1970s (Cooley et al. 1994). Cisplatin has demonstrated curative effects

for some cancers and has been instrumental in prolonging survival in other cancers (reviewed in Lebwohl and Canetta 1998). Furthermore, some of the nephrotoxicity associated with cisplatin can be offset by increased hydration and diuresis. However, like other chemotherapy drugs, resistance to cisplatin presents a major obstacle to treatment. This problem has prompted studies of other platinum-based drugs and combination therapy as alternative treatments for resistant cell types.

3.0 Cisplatin Overview

3.1 Use in Cancer Treatment

Today, cisplatin remains one of the most commonly used and effective chemotherapy drugs. It is a mainstay in the treatment testicular, ovarian, non-small cell lung, cervical, bladder, head and neck carcinomas, but has also been used to treat other cancers including colorectal carcinomas. Cisplatin is typically administered intravenously in several treatment cycles over a few weeks. The indiscriminate diffusion of this drug into most tissues of the body typically results in toxic side effects, particularly in the kidneys and intestine where cisplatin tends to concentrate (as reviewed in Cooley et al. 1994). Frequent side effects include kidney damage (nephrotoxicity) and gastrointestinal problems in the form of nausea, vomiting and electrolyte imbalances. Other less commonly encountered side effects include myelosuppression (decreased bone marrow function), liver damage, motor difficulties and sensory problems such as hearing loss, blurred vision and loss of taste (as reviewed in Von Hoff et al. 1979). Tumours which acquire resistance to cisplatin during the course of treatment require increasing

drug doses and consequently, patients experience more severe side effects. Resistance is therefore the most common reason for discontinuing treatment.

A number of other platinum-based molecules have been developed in an effort to find a drug with broader antitumour activity and fewer side effects than cisplatin. Among those currently in clinical use are carboplatin which is less toxic and remains in the bloodstream longer (Cemazar et al. 2006) and oxaliplatin which has been shown to achieve cytotoxic effects beyond that of cisplatin in some cell types (Faivre et al. 1999, Rave-Fränk et al. 2007). In contrast transplatin, an isomer of cisplatin, has relatively little cytotoxic activity in tumour cells which is thought to result from its inability to form 1,2-intrastrand DNA crosslinks due to steric hinderance (Eastman and Barry 1986).

3.2 Structure and Activity

Cisplatin is a square planar molecule with two chloride (Cl^-) and two ammine (NH_3^+) groups bound to a central platinum atom (Pt) in a *cis* configuration (see Figure 1). Although cisplatin binds with both DNA and other non-DNA targets such as membrane and plasma proteins, several pieces of evidence suggest that DNA is its primary target. Notably, cisplatin hypersensitivity has been reported in cells deficient in nucleotide excision repair, specifically, in cells with deficiencies in the transcription-coupled subpathway (Bulmer et al. 2005, Furuta et al. 2002). In addition, the relative inactivity of transplatin suggests that the 1,2-intrastrand DNA crosslink which it is unable to form may be an important contributor to the cytotoxicity of its isomer, cisplatin.

Cisplatin interacts irreversibly with DNA forming covalent bonds in a series of hydrolysis and nucleophilic substitution reactions. Activation of cisplatin depends on the concentration of chloride ions in the surrounding solution. Cisplatin is relatively inactive outside of cells (for example, in plasma) where the extracellular chloride concentration is ~100 mM; however, cisplatin is activated in the intracellular fluid where the chloride concentration is ~4 mM (Kartalou and Essigmann 2001a). At this lower concentration, the chloride ions are displaced by water molecules resulting in an aquated species $[Pt(H_2O)_2(NH_3)_2]^{2-}$ (Fuertes et al. 2003). Since water molecules make good leaving groups, the aquated species readily reacts with nucleophiles in the cell (Chu 1994). In the nucleus where cisplatin comes into contact with DNA, nucleophilic N7 atoms on exposed guanine and adenine bases react with the Pt atom displacing both water molecules to produce 1,2-d(GpG) intrastrand adducts (60-65% of crosslinks), 1,2-d(ApG) intrastrand adducts (20-25%), 1,3-d(GpXpG) intrastrand adducts (5-10%), d(G)/d(G) interstrand adducts (1-3%) and monofunctional adducts (see Figure 2) (Chaney et al. 2004). Interestingly, 1,2-intrastrand DNA crosslinks which make up the majority of cisplatin crosslinks are less efficiently repaired than 1,3-intrastrand crosslinks (Moggs et al. 1997). It is believed that this differential repair is due to the greater DNA helix distortion caused by 1,3-intrastrand crosslinks which are better recognized by the nucleotide excision repair system (Moggs et al. 1997).

3.3 Mechanism of Cytotoxicity

It is believed that the bulky DNA crosslinks formed by cisplatin result in the activation of cell death pathways. However, the exact mechanism(s) by which cisplatin DNA damage is coupled to cell death is not entirely clear. It has been suggested that DNA adducts interfere with transcription and/or DNA replication and failure of these processes triggers cell death. The notion that reduced transcription contributes to cytotoxicity is supported by observations of decreased RNA synthesis and stalled RNA polymerase II transcription in cells following cisplatin treatment (Corda et al. 1991, Mello et al. 1995). Transcription factors have also been found to bind Pt-GG intrastrand adducts with a high affinity leading to the hypothesis that adduct binding "hijacks" these molecules away from transcriptional activities (Jordan and Carmo-Fonseca 2000). In contrast, studies suggest that reduced DNA synthesis may not be linked to cisplatin cytotoxicity since cisplatin can induce cell death at concentrations too low to inhibit DNA synthesis and conversely, some cells are able to tolerate high doses which inhibit DNA synthesis (reviewed in Chu 1994).

It has also been suggested that persisting DNA damage in the transcribed strand of active genes resulting from the inefficient repair of cisplatin adducts may be a signal for cell death (Bulmer et al. 2005, Chu 1994). The observation that transplatin adducts (in contrast to cisplatin adducts), are repaired efficiently and have little cytotoxic activity supports this idea (Ciccarelli et al. 1985, Mello et al. 1995). Other lines of evidence include the observation of cisplatin hypersensitivity in repair-deficient cells and conversely, the identification of increased DNA repair as a mechanism of resistance in

some tumour cells. Several hypotheses have been proposed to explain why cisplatin adducts may not be repaired efficiently. One possibility is that platination levels in the genome may be too overwhelming for the cell machinery to repair. Another possibility is that proteins which bind cisplatin adducts may shield them from NER proteins; however, some investigators have found no evidence of adduct shielding (Moggs et al. 1997). A third possibility implicates the mismatch repair proteins and suggests that binding of these proteins to mismatched bases opposite cisplatin adducts may result in a futile repair process that is eventually aborted. A final hypothesis suggests that damage to mitochondrial DNA may trigger cell death since cisplatin is known to form a large number of adducts in mtDNA and mitochondria are unable to carry out excision repair (Fuertes et al. 2002). Given the important role of mitochondria in maintaining cellular ATP levels, damage to mtDNA may result in energy depletion and subsequent cell death.

4.0 Resistance to Cisplatin

4.1 Overview

Despite the effectiveness of cisplatin chemotherapy for a variety of tumours, some tumour cells show either intrinsic or acquired cisplatin resistance posing a major limitation to treatment. This has important consequences since even small changes in cell sensitivity to cisplatin (less that two-fold), can result in treatment failure. Therefore, extensive investigations have been carried out to determine the underlying cellular mechanisms of this resistance. A better understanding of these mechanisms will help to

identify cellular targets for modification and guide clinicians in choosing appropriate treatment regimens to circumvent resistance.

Resistance to cisplatin appears to be a multifactorial process and multiple mechanisms of resistance may be present in a single cell line. Such mechanisms include decreased intracellular drug accumulation, drug inactivation, altered apoptotic signaling and enhanced DNA repair. In cell lines where decreased intracellular drug accumulation has been observed, the reduction has been modest. At present, a specific membrane transport system for cisplatin has not been identified although it is known that the efflux pump encoded by the multi-drug resistance gene is not involved in this process (as reviewed in Kartalou and Essigmann 2001b). In other resistant cells, elevated levels of intracellular thiols like glutathione (GSH) and metallothionein (MT) have been observed. Both GSH and MT can bind multiple cisplatin molecules preventing their interaction with DNA targets (Ishikawa and Ali-Osman 1993, Kraker et al. 1985). Decreased expression of pro-apoptotic proteins (i.e. Bax, Bak, Bad) and increased expression of anti-apoptotic proteins (e.g. Bcl-2, Bcl-X_L) have also been identified in cisplatin resistant cell lines. For example, tumour cells with reduced levels of Bax and cells which have been transfected with Bcl-2 and Bcl-X_L show increased cisplatin resistance (as reviewed in Kartalou and Essigmann 2001b).

In addition to these mechanisms, a number of studies have implicated DNA repair as a key feature of resistance. Enhanced DNA repair has been observed in several cisplatin resistant cell lines as measured by loss of platinum adducts, DNA repair synthesis and reactivation of a cisplatin-damaged reporter gene (Shiebani et al. 1989,

Eastman and Schulte 1988, Cenni et al. 1999). Furthermore, while enhanced DNA repair has often been observed in resistant cell types, changes in drug uptake, intracellular drug inactivation and apoptotic signaling are not always observed. It has been suggested that increased DNA repair may be activated first in tumour cells followed by other induced mechanisms to achieve an even higher degree of resistance (Chu 1994). Studies have implicated the nucleotide excision repair, homologous recombination repair and mismatch repair systems in the enhanced repair capacity of cisplatin resistant cells (discussed below).

Rather than upregulating DNA repair in an error-free manner, some cells exhibit increased error-prone repair as a mechanism of resistance. Increased lesion bypass by DNA polymerases has been reported in a number of cisplatin resistant tumour cells (Mamenta et al. 1994, Vaisman et al. 1998). Consequently, these cells are able to tolerate greater amounts of DNA damage. The relative importance of lesion bypass versus error-free DNA repair to cisplatin resistance is not known, although the weight of the literature suggests that error-free repair (indicated by platinum removal from the genome), may be the more predominant repair mechanism.

4.2 Nucleotide Excision Repair and Resistance

The nucleotide excision repair (NER) pathway is involved in removal of damaged nucleotides from DNA and functions in maintaining genome integrity (Friedberg 2001). In the absence of NER, cells accumulate multiple genomic mutations through exogenous (environmental carcinogens) and endogenous (replication errors) processes, often leading

to cancer (Lodish et al. 2004). For example, Xeroderma Pigmentosum patients who are deficient for one or more NER proteins show a strong predisposition to skin cancer.

NER is the primary repair system for intrastrand cisplatin crosslinks which comprise the majority of cisplatin adducts. Figure 3 shows the two subpathways of NER, transcription-coupled repair (TCR) which involves preferential repair of transcribed strands in active genes (~30% of the genome) and global genome repair (GGR) which repairs both transcribed and nontranscribed strands in active and inactive genes (Furuta et al. 2002). The proteins involved in the initial recognition step of TCR and GGR differ, but then a common repair pathway is engaged (as reviewed in Costa et al. 2003, Furuta et al. 2002, de Laat et al. 1999). Briefly, repair-specific factors bind to the damaged DNA sites (these are CS-A and CS-B in TCR and XPC/hHR23B in GGR). Subsequently, TFIIH is recruited which contains two helicases XPB and XPD, that unwind a segment of DNA surrounding the damage. The open complex is then stabilized by XPA and RPA followed by endonuclease activity of XPG and XPF/ERCC1 which cleave the damaged strand at the 3' and 5' ends respectively (RPA, RF-C and PCNA proteins facilitate this excision). The gap is filled in by polymerases δ or ε and the newly synthesized strands are ligated to the remaining DNA with DNA ligase I. There is evidence to suggest that the TCR pathway is more critical to cell survival following cisplatin-induced DNA damage. TCR-deficient cells including Cockayne Syndrome groups A and B and Xeroderma Pigmentosum groups A, D, F, and G but not GGR deficient cells (Xeroderma Pigmentosum group C), show greater sensitivity to cisplatin (Bulmer et al. 2005, Furuta et al. 2002).

Enhanced NER has been reported in cisplatin resistant murine cells. Significantly, an increase in the repair of Pt-GG adducts was observed further alluding to the importance of this crosslink in cisplatin cytotoxicity (Eastman and Schulte 1998). Additionally, increased expression of the NER protein ERCC1 has been reported in resistant cell lines and in one study, disturbing the XPF/ERCC1 incision step of NER induced sensitivity in an initially resistant carcinoma cell line (Selvakumaran et al. 2003). Upregulation of other proteins involved in the NER pathway including XPF (Mukai et al. 2002) and XPE binding factor (Chu and Chang 1990) have also been reported in cisplatin resistant cells.

4.3 Homologous Recombination Repair and Resistance

Interstrand crosslinks which comprise up to 3% of cisplatin-induced adducts are repaired by homologous recombination repair (HRR). Excision of interstrand crosslinks results in double strand breaks and necessitates the use of sequence information from homologous chromosomes to resynthesize the missing DNA segments. HRR can proceed in cells as long as there is a homologous chromosome present and intragenic damage if present in both chromosomes, is non-overlapping. A number of proteins have been identified in the HRR pathway including, RAD51, RAD54, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, BRCA1 and BRCA2 (Xu et al. 2005). The precise roles of many of these proteins are not entirely clear although they are believed to operate as a functional unit and catalyze recombination by facilitating RAD51 assembly into nucleoprotein filaments (discussed below) (Bessho et al. 1997, Thompson and Schild

2001). Likewise, the proteins that initiate HRR have still to be elucidated. However, there is evidence for the involvement of the XPF/ERCC1 heterodimer in excision of interstrand crosslinks at the 5' end of the lesion (Bessho et al. 1997). Involvement of this complex in both NER and HRR may explain the extreme sensitivity of ERCC1 and XPF mutants to crosslinking agents (Bessho et al. 1997). The HRR pathway (reviewed in Lodish et al. 2004) is thought to begin with the generation of double strand breaks as an intermediate product (shown in Figure 4). Exonucleases are then activated which remove nucleotides from the 3' and 5' ends of the exposed strands to create single stranded 3' ends. RAD51 proteins (facilitated by BRCA1, BRCA2 and other proteins) form a nucleoprotein filament along the single stranded 3' ends. One RAD51 nucleoprotein filament searches for homologous DNA sequences on a sister chromatid and base pairs the damaged 3' single stranded end to the strand of the sister chromatid containing the complementary sequence. Using the strand from the sister chromatid as a template, DNA polymerases elongate the 3' end of the damaged DNA. The newly synthesized DNA segment then repairs with the other damaged 3' end and in turn acts as a template for DNA polymerases to fill in the gap on that strand. The newly synthesized DNA is then ligated to the surrounding DNA with DNA ligase.

Several studies have implicated HRR as a potentially important component of cisplatin resistance. Zhen et al. (1992) have reported that gene-specific repair of interstrand crosslinks but not intrastrand crosslinks was associated with cisplatin resistance in a human ovarian carcinoma line. Overexpression of the recombination repair protein XRCC3 in cells has been shown to result in increased cisplatin resistance

(Xu et al. 2005). In addition, knockout chicken cell lines with mutations in *Rad51b, Rad51c, Rad51d, Xrcc2 and Xrcc3* all exhibit impaired HRR and increased sensitivity to crosslinking agents such as cisplatin in comparison to cells expressing wildtype copies of these genes (Takata et al. 2000, Takata et al. 2001).

4.4 Mismatch Repair and Resistance

Several lines of evidence have implicated mismatch repair (MMR) deficiencies in resistance to cisplatin. Deficiencies in the MMR genes *hMLH1* and *hMSH2* also account for over 90% of hereditary nonpolyposis colorectal cancer (HNPCC) cases and a smaller proportion of sporadic colon cancers (Mitchell et al. 2002). It is believed that a germline mutation in one MMR allele is inherited and the second wildtype allele is subsequently inactivated resulting in tumourgenesis (Kolodner et al. 1995). Individuals with HNPCC are also predisposed to developing other types of cancer (i.e. ovarian, endometrial) at an early age. Tumours associated with HNPCC often demonstrate intrinsic resistance to cisplatin as a result of such MMR defects, posing a particular problem for clinical treatment.

The MMR system primarily recognizes and repairs mismatched bases, mispaired loops and frameshifts arising from errors in DNA replication, but also plays a role in regulating recombination repair. There are five principal MMR proteins involved in eukaryotic repair, MLH1 and PMS2 (homologues of the mutL bacterial protein) and MSH2, MSH3 and MSH6 (homologues of the mutS bacterial protein). The MMR pathway involves i) damage recognition ii) repair complex stabilization iii) DNA

excision and iv) DNA resynthesis as seen in Figure 5 (reviewed in Jascur and Boland 2006). Briefly, MSH2 binds with either MSH6 or MSH3 constituting MutSα and MutSβ damage recognition complexes respectively. MutSα recognizes single base mismatches and small insertion/deletion loops whereas MutSβ recognizes larger insertion/deletion loops. Binding of both complexes to DNA is driven by ATP. The initial recognition complex is stabilized by the MLH1/PMS2 heterodimer (MutLα), which acts as a "molecular matchmaker" removing replication proteins from nascent strands containing errors and recruiting Exonuclease I (ExoI) to the damaged site. ExoI excises the mismatched bases in a 5' to 3' manner (excision of mismatches in a 3' to 5' manner are thought to involve the MutLα complex, as reviewed in Yang 2007) and RPA stabilizes the remaining single strand and helps to terminate the excision process. DNA resynthesis is completed by DNA polymerase δ (in collaboration with PCNA which enhances its processing activity); however, there is some evidence for accessory roles of DNA polymerases α and ε in this step.

Recognition of base mismatches among the millions of other normal base pairs is thought to depend on kinks created in DNA at mismatch sites due to base unstacking. Yang (2006) has proposed a model for mismatch recognition whereby sites of unstacked bases (flexible hinges) may be preferentially scanned by the MMR system and nucleotides which do not form a normal Watson-Crick base pair are subsequently identified upon further inspection by the recognition complex. Criteria such as the shape, hydrogen bonding and electrostatic potentials of the bases at these sites appear to be critical to mismatch recognition. While strand specificity for MMR proteins in

prokaryotes is conferred by an endonuclease (MutH) which preferentially nicks the unmethylated daughter strand, eukaryotes lack a similar endonuclease that targets the nascent strand for repair. It is believed that Okazaki fragments which create discontinuities in the nascent strand may serve as a signal for strand specificity, similar to the nicks created by the MutH protein in prokaryotes (as reviewed in Yang 2007).

Increasingly, it appears that the MMR system not only functions in maintaining genome integrity but also participates in other cellular processes. Components of the MMR system may have a broader role than previously thought in sensing DNA damage and multiple lines of evidence suggest that MMR proteins are involved in the recognition of cisplatin-damaged DNA. The bacterial MutS protein shows selective, weak binding to the major cisplatin intrastrand Pt-GG adduct; however, the presence of a mispaired base opposite the lesion increases binding efficiency for both Pt-GG and Pt-AG adducts (Fourrier et al. 2003). Similarly, the human MutSα heterodimer has been shown to bind Pt-GG adducts (Duckett et al. 1996). Furthermore, hMSH2 shows selective binding to DNA adducts of platinum compounds with therapeutic efficacy but not DNA modified by ineffective platinum compounds (Mello et al. 1996).

Several studies have demonstrated a positive correlation between MMR deficiency and cisplatin resistance suggesting that binding of MMR proteins to cisplatin adducts may mediate the drug's cytotoxic effects. Specifically, investigators have shown that hMLH1-deficient, hMSH2-deficient and hMSH6-deficient human adenocarcinoma cells are more resistant to cisplatin than their MMR proficient counterparts (See Abei et al. 1996, Cenni et al. 1999, Fink et al. 1996, Vaisman at al. 1998). In contrast, defects in

hMSH3 do not appear to affect cisplatin resistance suggesting that hMutSβ may play only a minor role in damage recognition or binding of this recognition complex may not adversely affect processes that give rise to resistance (Vaisman et al. 1998). Similarly, cisplatin resistance has been demonstrated in murine MSH2 and PMS2 knockout cell lines which provide a more robust test of the contribution of MMR deficiency to resistance (Fink et al. 1997). Few studies have examined repair of cisplatin-damaged DNA in MMR-deficient versus MMR-proficient cell lines (most published studies have utilized clonogenic survival assays or growth rate assays to assess resistance). However, one study employing a host cell reactivation assay demonstrated that MMR-deficient HCT116 and HEC59 cells were two-fold more efficient in repairing a cisplatin-damaged reporter gene than wildtype controls in which MMR activity was restored with chromosome transfection (Cenni et al. 1999). Examination of the repair capacity in other MMR-deficient cell lines warrants further attention.

The mechanism by which loss of MMR in cells contributes to cisplatin resistance is not clear; however, a number of hypotheses have been suggested. The currently accepted hypothesis is the "futile repair" hypothesis which is based on observations of futile cycles of MMR at DNA sites opposite bases modified by methylating agents. During this process an incorrect thymine base is continually misincorporated across from methylated bases in the template strand, leading to continual removal and resynthesis of the misincorporated base (Jascur and Boland 2006). Likewise, a similar process is thought to occur opposite cisplatin lesions in the template strand leading to strand breaks, aborted repair and apoptosis. A second hypothesis suggests that binding of MMR

proteins to cisplatin lesions may lead to processing of the strand in a way that impairs repair by NER proteins (i.e. damage to the template strand) (Cenni et al. 1999). A third hypothesis suggests that binding of MMR proteins to cisplatin lesions prevents bypass of the lesion by DNA polymerases. Studies have shown that cells deficient in hMLH1 and hMSH6 show increased replicative bypass of cisplatin adducts (Vaisman et al. 1998); however, MMR deficiency is not a prerequisite for this process suggesting that avoidance of futile repair or adverse DNA strand processing may be more critical to resistance in MMR-deficient cells.

5.0 UVC Irradiation

5.1 Overview

Like the effects of cisplatin, chemical modification to DNA can also occur through exposure to ultraviolet (UV) radiation. Solar radiation from the sun is the primary environmental source of UV radiation. UV radiation can be divided into three wavelengths, UVA (320-400 nm), UVB (280-320 nm) and UVC (<280 nm) (Matsumura and Ananthaswamy 2004). UVA and UVB radiation are thought to be the most relevant to biota since the shorter wavelength UVC radiation is generally absorbed into the ozone layer (Sinha and Häder 2002). UVB radiation is the more damaging of the two wavelengths that reach the Earth's surface since it is absorbed by DNA, resulting in cellular damage to living organisms. In contrast, UVA radiation is not absorbed by DNA, but can indirectly cause damage to cells through the formation of reactive oxygen species (Sinha and Häder 2002).

Absorption of UV radiation by DNA results in two types of DNA distorting lesions: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts, both of which occur between adjacent thymine and cytosine bases (see Figure 6) (Matsumura and Ananthaswamy 2002). CPDs occur more frequently in DNA than 6-4 photoproducts and they are repaired more slowly; therefore, CPDs are thought to be more mutagenic lesions (Mitchell et al. 1985).

5.2 Repair of UV-induced DNA Damage

If left unrepaired, UV damage can result in DNA mutations. The most typical mutations resulting from UV lesions are C to T or CC to TT base transitions which are believed to arise due to the default insertion of adenine bases opposite CPDs and 6-4 photoproducts (Matsumura and Ananthaswamy 2004). When the altered DNA sequences containing the adenine bases are replicated, point mutations are created. Like cisplatin lesions, UV lesions are primarily repaired by NER. UV-induced dimers are recognized, excised and the gap is filled in by DNA polymerases. However, an important difference between the repair of cisplatin and UV lesions is that the latter is less dependent on recombination repair since interstrand DNA crosslinks are less frequent following UVC exposure and double-strand DNA breaks are not typically associated with UV-irradiation (Friedberg et al. 1995). This difference is important to keep in mind when comparing the effects of UV-induced and cisplatin-induced DNA damage in cells.

5.3 Deficiencies in the Repair of UVC-induced DNA Damage

Two diseases, Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS) are characterized by defects in NER and consequent hypersensitivity to UV radiation. Associated symptoms of both diseases include excessive pigmentation of the skin and premature skin aging however, only patients with XP show an increased risk of skin cancer with an early age onset (Matsumura and Ananthaswamy 2002). Depending on the XP protein affected, XP patients may show deficiencies in both subpathways of NER or the GGR subpathway only whereas, CS patients show deficiencies in the TCR subpathway alone. Therefore, in XP patients there is greater opportunity for the accumulation of UV-induced lesions in the genome, making it more likely that a given mutation will result in the initiation of cancer. XP patients are predisposed to developing skin cancers such as basal cell carcinomas, squamous cell carcinomas and melanomas and have an increased risk of developing internal cancers by 20 years of age (Matsumura and Anathaswamy 2002).

6.0 Hypoxia and the Tumour Microenvironment

6.1 Tumour Physiology and Adaptive Changes

The tumour microenvironment is characterized by transient and/or chronic hypoxia (low oxygen tension), nutrient deprivation, decreased ATP levels, low pH and acidosis. This microenvironment results from an imbalance in oxygen supply and tissue demand in solid tumours. Rapidly growing tumours outstrip their blood supply so that oxygen must diffuse over increasingly greater distances to supply tumour tissues. This

results in areas of hypoxia. However, tumour cells under hypoxic stress undergo a number of adaptive changes to counteract this reduced oxygen tension. These changes are mediated by hypoxia-inducible factor-1 (HIF-1) consisting of the subunits HIF-1α which is stabilized under hypoxic conditions and HIF-1β which is constitutively expressed in cells (Leo et al. 2004). HIF-1 binds to DNA sequences known as *hypoxia response elements* resulting in the activation of genes that promote anaerobic glycolysis, enhanced oxygen delivery (i.e. angiogenesis, increased hemoglobin) and pH regulation (Leo et al. 2004). In the case of angiogenesis (outgrowth of microvessels from existing blood vessels), it has been shown that this directed growth often results in a highly disorganized network of vessels with severe structural and functional abnormalities (Vaupel 2004). The limited perfusion of oxygen into these abnormal microvessels contributes further to oxygen deprivation in tumours. In normal tissues such adaptive changes would be able to reestablish normoxic conditions; however, in tumourgenic tissues these changes are not sufficient to overcome the oxygen deficit so a chronic state of hypoxia develops.

6.2 Hypoxia and Cell Survival

The effects of chronic hypoxia appear to be cell specific. In some cancer cells hypoxia induces cell death whereas in other cells it appears to have a protective effect (Wang et al. 2006, Yao et al. 2005). Hypoxia may slow down or inhibit cell proliferation in some tumour cells and drive them into G1/S arrest; however, the extent of this inhibition may depend on the concurrence of other adverse microenvironmental

conditions such as acidosis (Vaupel 2004). In addition to reducing cell proliferation hypoxia may also induce cell apoptosis through p53-dependent and -independent pathways. In contrast, hypoxia may increase the survival of tumour cells by inducing changes that increase cell proliferation or protect cells from apoptosis resulting in resistance to therapeutic agents (Yao et al. 2005). Such changes may include the upregulation of genes which positively regulate the cell cycle or promote metastasis (i.e. growth factors, proteins which increase cell mobility). Alternatively, hypoxia may select for cells that have lost the ability to undergo apoptosis or those with a more aggressive phenotype. Hypoxia has also been shown to upregulate anti-apoptotic proteins in some tumour cells (Leo et al. 2004). Therefore, both pro-apoptotic and anti-apoptotic signals may be induced by hypoxia and the decision to live or die will likely depend on the outcome of crosstalk between multiple intracellular signals as well as the specific tumour cell.

6.3 Hypoxia and DNA Repair

Other hypoxic effects in cells have been observed including alterations in the expression of DNA repair genes and genetic instability (e.g. increased mutations, gene amplification). Notably, downregulation of several genes involved in MMR and recombination repair has been reported under conditions of hypoxia. These findings have important implications for repair of cisplatin-damaged DNA considering the roles MMR and HRR proteins in the recognition and repair of such damage. Mihaylova et al. (2003) have shown decreased expression of *hMLH1* at the mRNA level and a corresponding

post-transcriptional decrease in its heterodimer partner hPMS2 in hypoxic cells. Increased mutagenesis was also noted in these cells suggesting that reduced MMR activity under hypoxic conditions may contribute to genetic instability. Similarly, decreased expression of *hMSH2* and *hMSH6* in a HIF-1α and p53-dependent manner has been reported in hypoxic cells with corresponding increases in microsatellite instability (Koshiji et al. 2005). It should be noted however that the specific MMR genes downregulated in hypoxia varies between cell lines and may depend on the p53 status of the cell. More recently, decreased expression of *RAD51* was demonstrated in hypoxic cells and this transcriptional repression corresponded to a functional decrease in HRR (Bindra et al. 2005). Furthermore, this downregulation of *RAD51* appears to be HIF-1α and p53-independent.

Few studies have examined NER in hypoxic cells. However, one important study found that HCR of a UV-damaged reporter gene was reduced in murine 3340 cells and human RCneo cells under conditions of hypoxia and low pH (Yuan et al. 2000). This study also reported elevated mutability in these hypoxic cells. These results suggest that decreased DNA repair in hypoxic cells may facilitate the accumulation of genetic mutations leading to genetic instability. However, little is known about the effects of hypoxic conditions on the repair of cisplatin-induced DNA damage and this may differ from the effects of hypoxia on the repair of UV-damaged DNA in important ways.

7.0 Measuring DNA Repair and Cell Sensitivity to Cytotoxic Agents

7.1 Host Cell Reactivation Assay

The host cell reactivation assay (HCR) examines the ability of cells to repair (or "reactivate") damaged exogenous DNA rather than their own DNA. The repair of various types of exogenous DNA including bacteriophage DNA, mammalian viral DNA and plasmid-borne or viral-encoded reporter genes has been examined in different cell types (reviewed in Friedberg et al. 1995). In the present work, we have looked at the ability of cells to repair a damaged *lacZ* reporter gene encoded by a recombinant non-replicating viral construct. The viral construct is first treated with a DNA damaging agent and then transfected into cells. A chemical compound such as Chlorophenolred-β-D-galactopyranoside (CPRG) which reacts with β-galactosidase (the *lacZ* protein product) is then added to cells producing a colour change. The extent of reporter gene reactivation is therefore indicated by the degree of this colour reaction as measured by a spectrophotometer.

There are other ways in which DNA repair can be quantified apart from a HCR assay (including measurement of newly synthesized DNA and quantifying the removal of DNA lesions). However, there are several advantages to using a reporter gene-based HCR assay over these other DNA repair methods. First, HCR is a sensitive indicator of DNA repair capacity in host cells and is a relatively easy assay to perform (Friedberg et al. 1995). Second, HCR assays can measure both constitutive repair as well as induced repair since the viral construct and cell can be treated separately with DNA damaging agents. Third, this assay looks at repair of actively transcribed genes and measures a

functional protein product as an endpoint; therefore, the ability of cells to successfully complete all steps in repair (but possibly also lesion bypass), is evaluated (Cenni et al. 1999). Fourth, the HCR assay has been previously validated as a method for measuring repair of both cisplatin-induced and UV-induced DNA lesions (Cenni et al. 1999, Parker et al. 1991, Sheibani et al. 1989, Smith et al. 1995, Yuan et al. 2000).

An inherent assumption with the HCR assay we have used is that β-galactosidase (β-gal) expression requires the removal of transcription-blocking lesions from the *lacZ* gene. However, another possibility which cannot be ruled out completely (at least for cisplatin-induced damage), is that RNA polymerases may bypass the lesion and generate silent mutations allowing a functional protein to be expressed (Cenni et al. 1999). Using a QPCR technique it has been shown previously that UVC-induced lesions are removed from the *lacZ* gene in AdHCMV*lacZ* in normal but not in NER-deficient XP and CS cells (Boszko and Rainbow 1999) and this correlates with HCR of β-gal expression, providing evidence that HCR of β-gal expression reflects repair of the *lacZ* gene for UVC damage. The removal of cisplatin-induced lesions from the *lacZ* gene has not been examined, but the UVC data above suggest that HCR of β-gal activity for the cisplatin-treated *lacZ* gene may reflect lesion removal.

It should also be noted that with a HCR assay the DNA conformation and/or chromatin structure of the exogenous viral construct may differ to that in the cell possibly resulting in small differences in the recognition and repair of DNA damage in viral DNA versus cellular DNA (Rainbow et al. 2005).

7.2 Clonogenic Survival Assay

The clonogenic survival assay examines the sensitivity of cells to various DNA damaging agents. In this procedure, cells are plated at a low density, treated with a DNA damaging agent and surviving cells are allowed to divide forming colonies. This assay most closely corresponds with the clinical responses of cells to cancer treatment since colony formation is indicative of cells whose replication machinery remain intact following exposure to cytotoxic agents (Hall 2000). However, this assay is limited as a tool for assessing the sensitivity of primary cells given their poor plating efficiency (Hall 2000).

Cell survival at various treatment doses is expressed relative to untreated controls. This ratio is referred to as the *surviving fraction.* D_{37} is the dose that corresponds to a surviving fraction of 0.37 and is used as a standardized measure of sensitivity to cytotoxic agents. There as an inverse relationship between the D_{37} value and cell sensitivity. Generally, the greater the D_{37} value the lower the cell sensitivity to the DNA damaging agent and vice versa.

8.0 Research Project and Objectives

Cisplatin resistance remains an elusive problem in the treatment of tumours. An understanding of the underlying mechanisms of resistance is a necessary step towards predicting clinical responses to cisplatin and improving treatment strategies to circumvent resistance. Several reports suggest that one aspect of resistance may involve the loss of MMR protein function (Abei et al. 1996, Cenni et al. 1999, Fink et al. 1996, Fink et al.

1997, Vaisman et al. 1998). However, contradictory reports suggest that loss of MMR alone may not be able to account for resistance (Claij and te Riele 2004) and it has been suggested that other concurrent mutations may be requisite for resistance (Branch et al. 2000, Sansom and Clarke 2002). Additionally, hypoxia has been shown to influence cellular responses to DNA damaging agents, specifically UV-irradiation (Yuan et al. 2000). However, little is known about the repair of cisplatin-damaged DNA in hypoxia.

The current research aims to further clarify the role of MMR in cisplatin resistance and examines the possible contributions of other specific cellular changes to tumour resistance. This research also aims to extend the literature on the effect of hypoxia on repair of cisplatin-damaged DNA given that repair *in vivo* occurs in the context of a hypoxic tumour microenvironment.

In chapter 2, we have examined repair of cisplatin-damaged DNA and cisplatin sensitivity in isogenic murine fibroblasts under normoxic and hypoxic conditions. We have used an HCR assay to examine repair and a clonogenic survival assay to examine sensitivity. The use of isogenic murine fibroblasts with knockout and wildtype copies of various MMR proteins provided a well defined system for determining the precise role of MMR in cisplatin resistance. Furthermore, the inclusion of both primary and transformed fibroblasts allowed for a comparison of the effects of transformation-induced changes on cell repair and sensitivity.

In chapter 3, we extended our investigation to include an evaluation of the effects of other cell alterations (i.e. acquired mutations, transformation-induced changes) and hypoxic effects on the repair of cisplatin-damaged DNA in several human cell lines. We

examined differences in repair under normoxic and hypoxic conditions in p53-deficient Li-Fraumeni cells, SV40-transformed normal human fibroblasts and primary normal human fibroblasts. In addition, we examined repair in MMR-deficient and -proficient human tumour cells under conditions of normoxia and hypoxia. We sought to determine whether we could predict responses to cisplatin in these tumour lines based on our understanding of the effects of MMR loss, p53 deficiency, SV40-transformation and hypoxia on repair of cisplatin-damaged DNA.

In chapter 4, we have compared the repair of cisplatin-damaged DNA to that of UV-damaged DNA in normal human cells under normoxic and hypoxic conditions. NER is the primary repair pathway for both types of damage. However, HRR is also involved in the repair of cisplatin damage. We sought to determine whether the effects of hypoxia on repair of both kinds of DNA damage would be similar due to the common engagement of the NER pathway or different as a result of the additional involvement of HRR for cisplatin lesions. We have also investigated the effects of hypoxia and low pH on DNA repair since the occurrence of both conditions more accurately mimics the *in vivo* tumour microenvironment where these factors may interact in important ways. Additionally, it has been suggested that both conditions may be necessary to bring out repair differences in cells (Yuan et al. 2000).

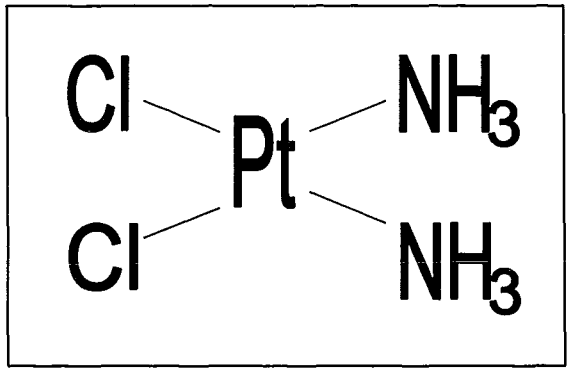

Figure 1. Chemical structure of cisplatin

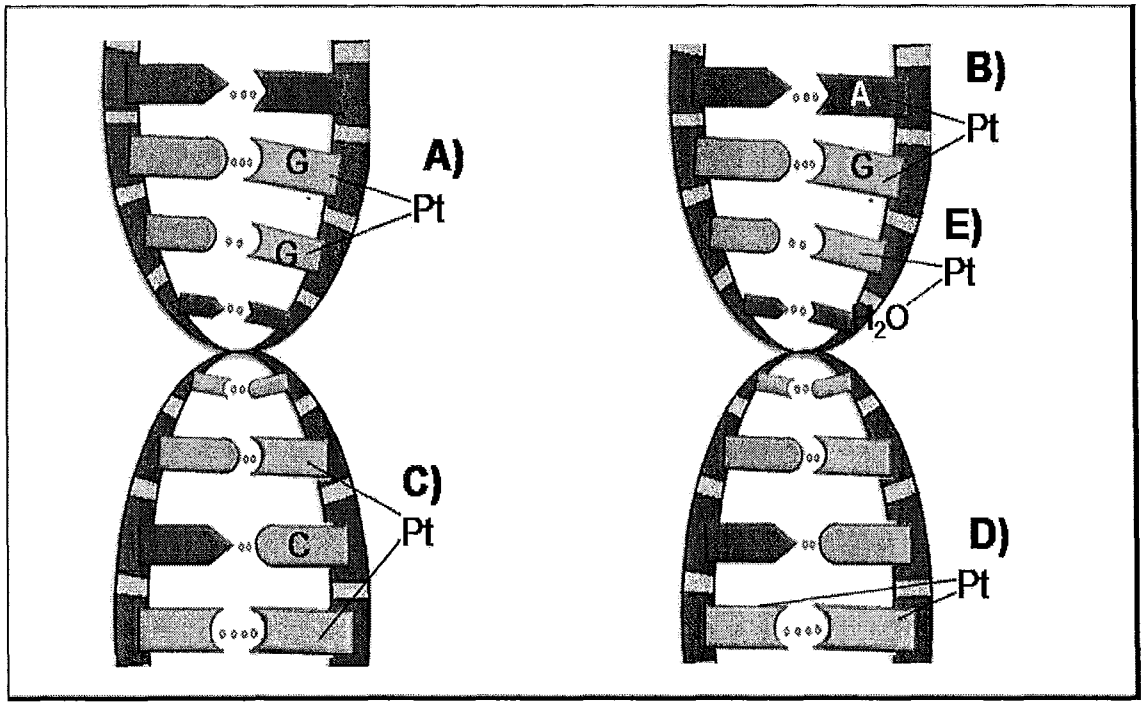

Figure 2. Examples of cisplatin adducts. A) 1,2-d(GpG) intrastrand adduct B) 1,2-d(ApG) intrastrand adduct C) 1,3-d(GpG) intrastrand adduct D) d(G)/(G) interstrand adduct E) monofunctional adduct (DNA image adapted from Muskopf 2007).

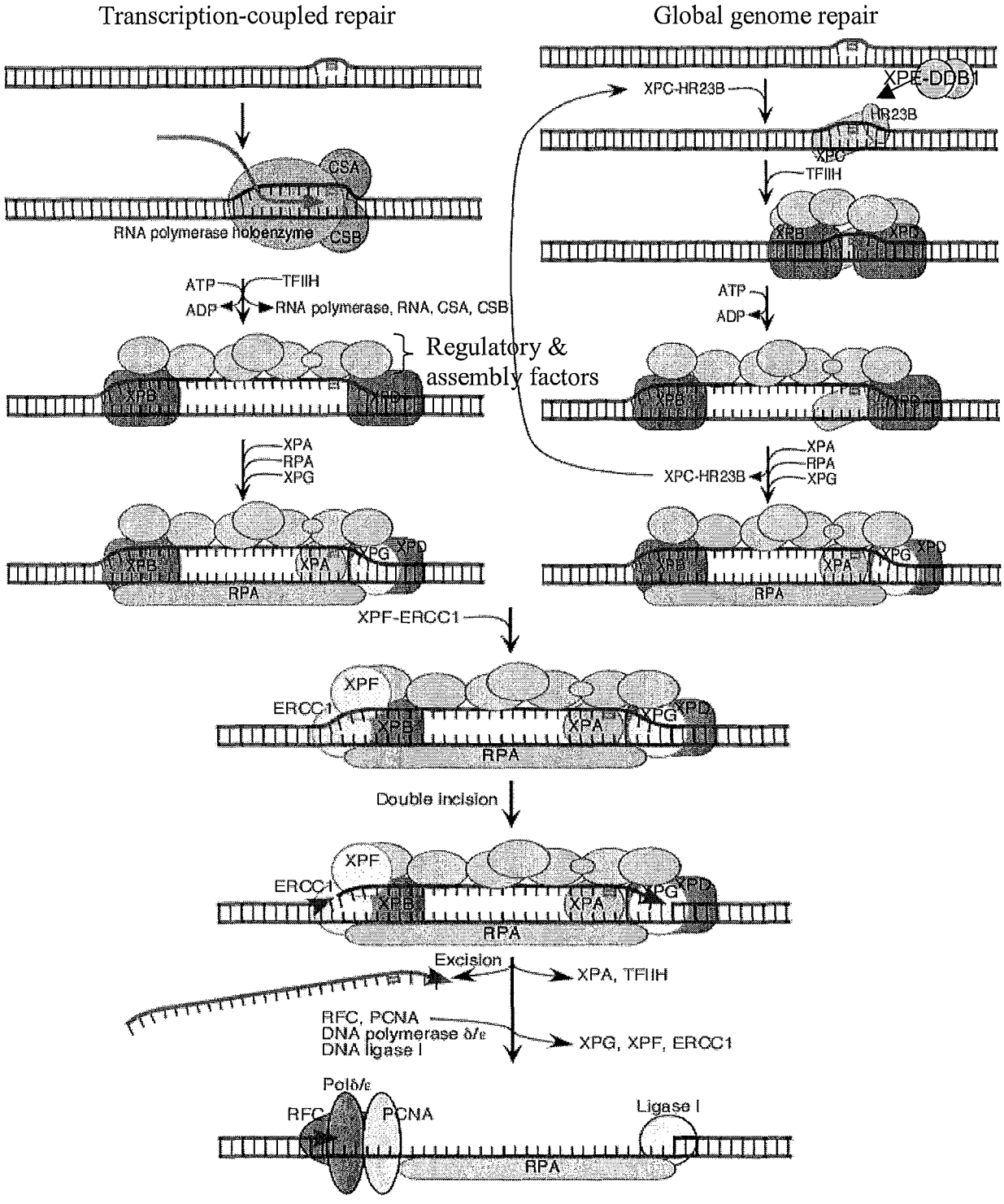

Figure 3. Schematic of the eukaryotic nucleotide excision repair pathway (adapted from Huberman 2006). Top left panel: initiation of transcription-coupled repair subpathway. Top right panel: initiation of global genome repair subpathway.

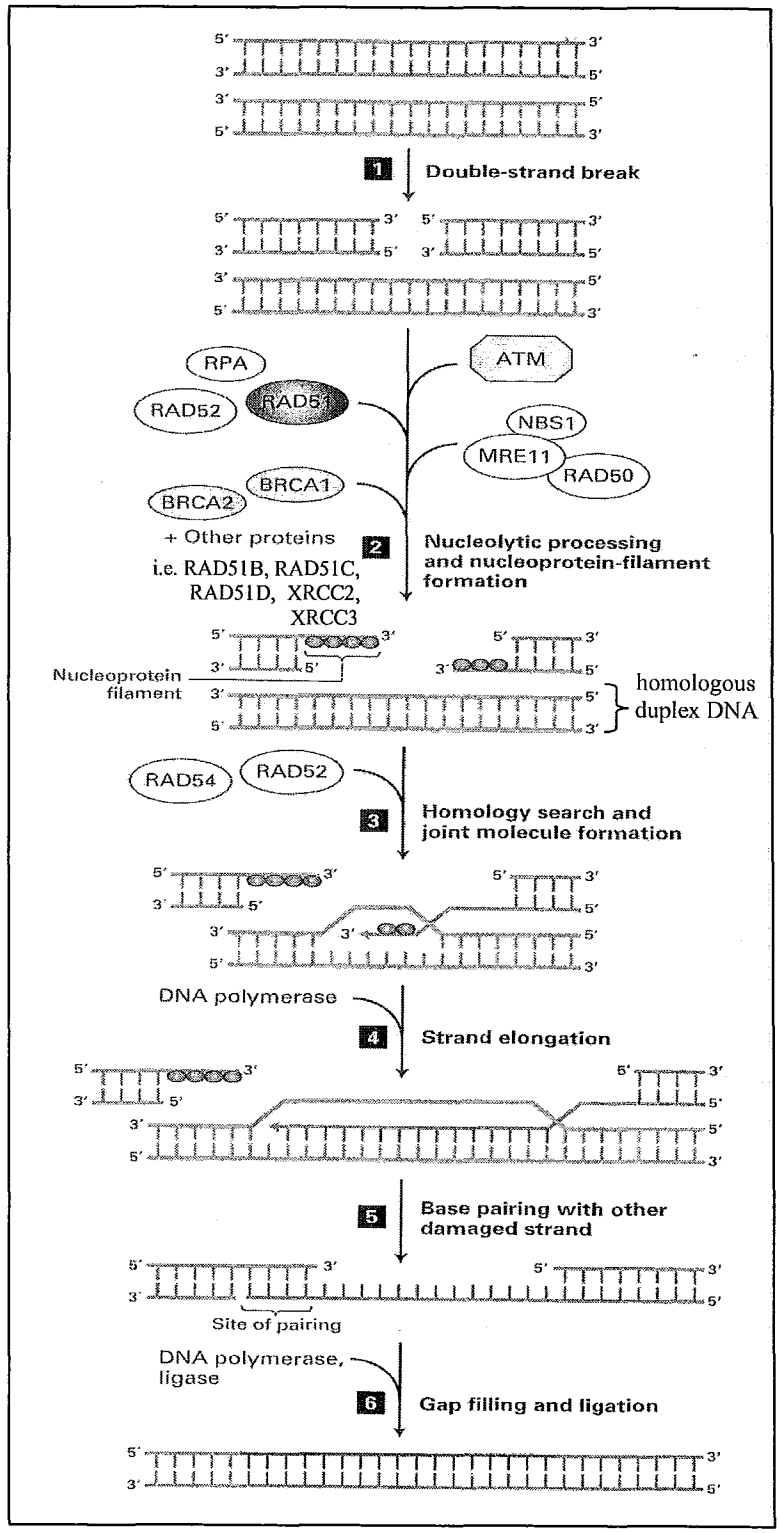

Figure 4. Schematic of the homologous recombination repair pathway (adapted from Lodish et al. 2004)

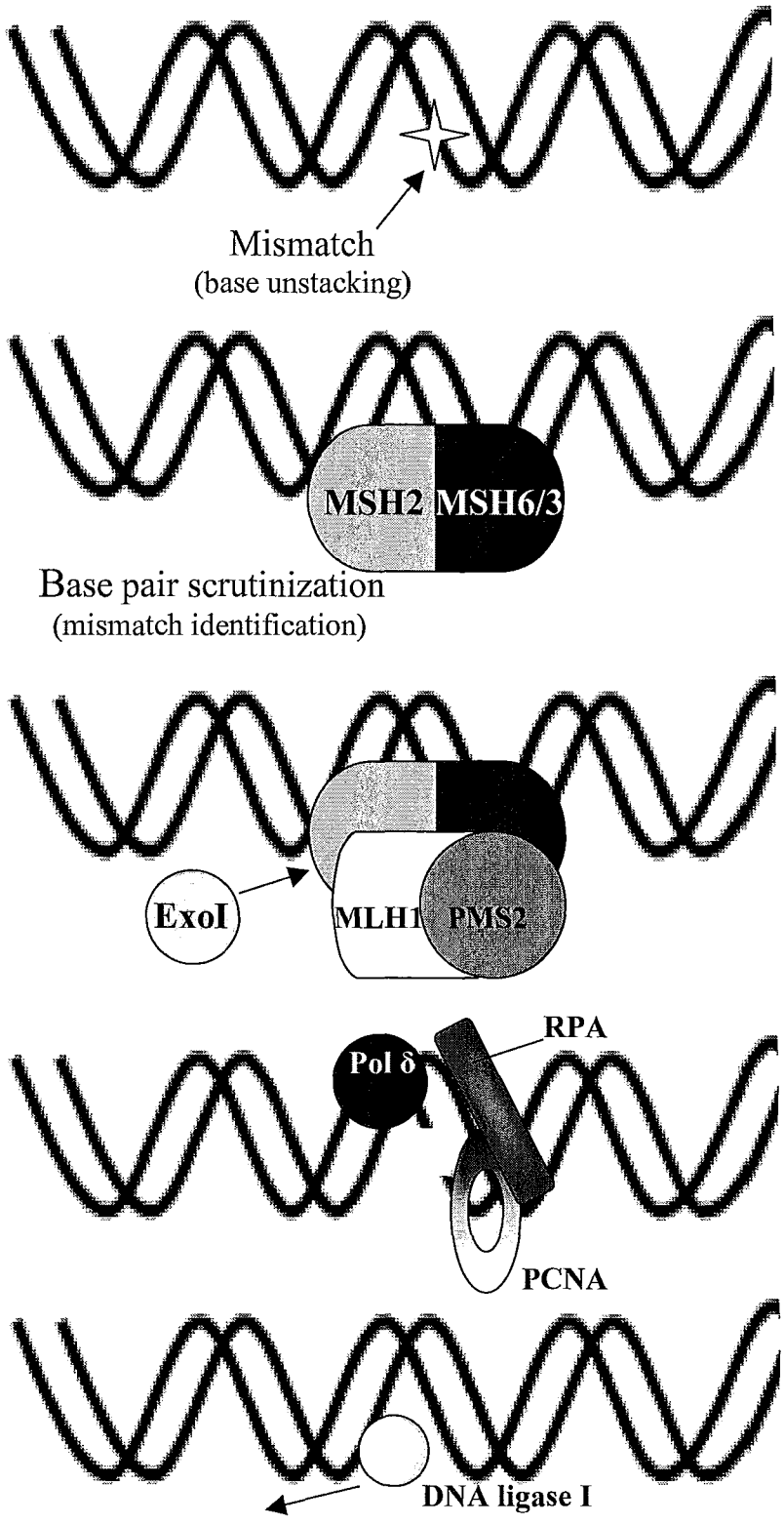

Figure 5. Schematic of the eukaryotic mismatch repair pathway

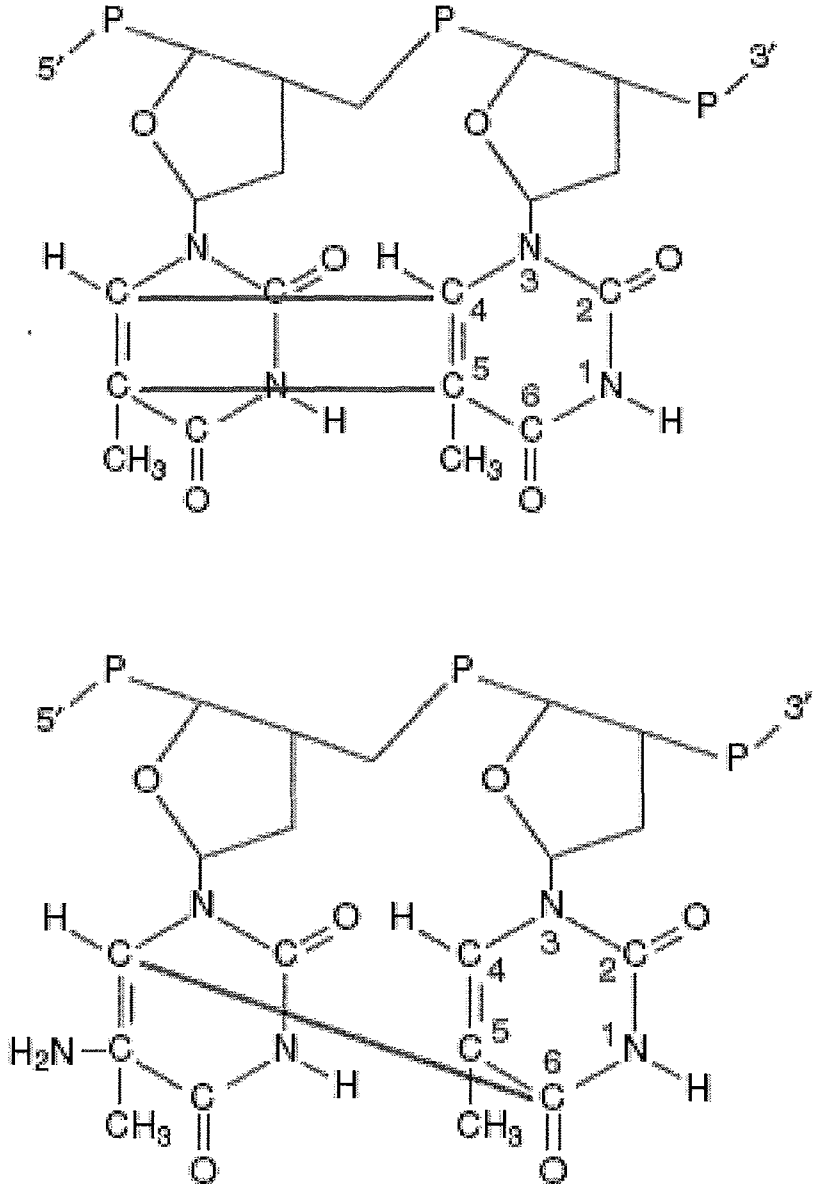

Figure 6. Examples of UV-induced DNA damage (from Matsumura and Ananthaswamy 2002). Top: cyclobutane pyrimidine dimer. Bottom: 6-4 photoproduct.

CHAPTER 2

Effects of Mismatch Repair Status and Hypoxia on Cisplatin Sensitivity and Repair of Cisplatin-damaged DNA in Murine Embryonic Fibroblasts

1.0 Abstract

Mutations in the human mismatch repair (MMR) genes are associated with hereditary nonpolyposis colorectal cancer as well as other sporadic cancers (Fink et al. 1997). MMR gene mutations have also been implicated in the resistance of human tumours to cisplatin and several tumour-derived MMR-deficient cells show cisplatin resistance *in vitro*. However, reports concerning the cisplatin resistance of MMR-deficient murine cells *in vitro* are conflicting (Fink et al. 1997, Claij and te Riele 2004). In addition, hypoxia, a common feature of the tumour microenvironment has been shown to influence tumour responses to conventional cancer treatments. We have examined the role of MMR proteins and hypoxia on cisplatin sensitivity and repair of cisplatin-damaged DNA in MMR-deficient murine fibroblasts and MMR-proficient controls. A host cell reactivation (HCR) assay employing a non-replicating recombinant virus expressing the β-galactosidase reporter gene was used to measure repair of cisplatin-damaged DNA. Sensitivity to cisplatin was measured using a clonogenic survival assay. HCR of the cisplatin-damaged reporter gene was significantly greater in SV40-transformed MSH2-deficient cells compared to MSH2-proficient controls under normoxic conditions. In contrast, HCR was similar in primary MSH2-deficient and MSH2-proficient cells. Repair was also similar in primary MSH6-deficient and MSH6-proficient cells as well as in SV40-transformed PMS2-deficient and PMS2-proficient cells. These results suggest that loss of MMR alone does not result in increased repair of cisplatin-damaged DNA and additional concomitant alterations in tumour and SV40-transformed cells are responsible for cisplatin resistance through a modulation of repair

capacity. Hypoxia treatment 24 h pre-infection and 40 h post-infection reduced HCR in both primary MSH2-deficient cells and transformed PMS2-deficient cells compared to normoxia. In contrast, repair was enhanced under hypoxic conditions in primary MSH2-proficient cells. HCR was also reduced in MSH6-proficient cells under hypoxic conditions at 24 h post-infection. These results suggest that the hypoxic tumour microenvironment may influence the outcome of cisplatin therapy through a modulation of repair. Finally, survival of SV40-transformed MSH2-deficient cells was significantly greater than that of MSH2-proficient controls following cisplatin treatment suggesting that the enhanced repair observed in transformed MSH2-deficient cells contributes to cellular resistance. Survival of SV40-transformed MSH2-deficient cells was also greater under hypoxic versus normoxic conditions. A previous report has shown diminished DNA repair and elevated mutagenesis under conditions of hypoxia following UV exposure (Yuan et al. 2000). It is therefore possible that reduced repair of cisplatin-damaged DNA coupled with increased survival in MSH2-deficient cells under hypoxic conditions may lead to elevated mutagenesis following cisplatin treatment.

2.0 Introduction

Cisplatin is one of the most effective and commonly used drugs for cancer chemotherapy. It has demonstrated anticancer effects through the formation of bulky DNA crosslinks for a broad spectrum of solid tumours. However, intrinsic and acquired resistance to cisplatin has been observed in a number of cancers posing a major challenge to clinical treatment. Mutations in human MMR genes *hMLH1*, *hMSH2* and to a lesser

extent *hPMS2* account for 90% of hereditary nonpolyposis colorectal cancer and *hMSH6* mutations are associated with other sporadic cancers (as reviewed in Fishel and Kolodner 1995, Fink et al. 1997). Defects in MMR genes have been implicated in the resistance of human tumour cells to cisplatin *in vitro* (Cenni et al. 1999, Fink et al. 1997 and Vaisman et al. 1998). However, reports of cisplatin resistance in MMR-deficient murine cells *in vitro* are conflicting and suggest the joint requirement of MMR deficiency and additional gene alterations for cisplatin resistance. For example, Claij and te Riele (2004) found that the responses of MSH2-deficient and MSH2-proficient mouse embryonic stem cells to cisplatin were similar and restoration of *Msh2* activity in deficient cells with cDNA did not increase their sensitivity. In addition, Sansom and Clarke (2002) have reported only a slightly reduced apoptotic response in MSH2-deficient murine intestinal enterocytes which had no overall effect on survival following cisplatin treatment.

The tumour microenvironment represents another important influence over cellular responses to DNA damaging agents. This microenvironment results from an inadequate blood and oxygen supply and is characterized by regions of fluctuating or chronic hypoxia, low pH and nutrient deprivation. Studies have shown that cells under hypoxic stress exhibit decreased DNA repair, elevated mutagenesis, downregulation of key repair proteins and abnormal cell physiology (Mihaylova et al. 2003, Yuan et al. 2000).

Since tumour cell responses to cisplatin depend on nucleotide excision repair (NER), we have used a HCR assay to examine the effects of MMR loss and hypoxia on the repair of cisplatin-damaged DNA in MMR-proficient and -deficient isogenic murine

embryonic fibroblasts (MEFs). We hypothesized that loss of MMR alone may not be able to account for resistance to cisplatin and resistance may require other concomitant cell alterations.

The use of primary and transformed isogenic murine fibroblasts allowed for a more precise determination of the role of MMR in resistance to cisplatin and circumvented some of the difficulties associated with human tumour cells such as additional acquired mutations and chromosome transfer to restore wildtype MMR activity. Additionally, few studies have compared the repair of cisplatin damage in primary versus transformed fibroblasts. Changes induced by transformation with simian virus 40 (SV40) may have important effects on DNA repair.

In addition, we have examined the sensitivity of SV40-transformed MSH2-deficient and -proficient MEFs to cisplatin using a clonogenic survival assay. We sought to determine whether increased repair of cisplatin-damaged DNA in these MMR-deficient cells was concomitant with increased cell survival as several studies have implicated DNA repair as a key mechanism of cisplatin resistance (as reviewed in Chu 1994). We also sought to determine whether differential effects of hypoxia on repair of cisplatin-damaged DNA in MSH2-deficient and MSH2-proficient MEFs could account for increased mutability, since increased mutagenesis has been reported for some cell types under conditions of hypoxia (Yuan et al. 2000).

3.0 Materials and Methods

3.1 Cell Lines

Primary MEFs with knockout and wildtype copies of *Msh2* and *Msh6* and transformed MEFs with knockout and wildtype copies of *Msh2* and *Pms2* were used in the current experiments. All cell lines are described in Table I. MMR-deficient M2KO, M6KO and MS5-7 cells and their MMR-proficient counterparts M2WT, M6WT and BCl-6 cells respectively, were obtained from Dr. S.E. Andrew at the University of Alberta and have been described previously (Fritzell et al. 1997). SV40-transformed PMS2-deficient and -proficient MEFs (334029 and 3340Cl1 respectively), were provided by Dr. P. Glazer at Yale University School of Medicine, New Haven, Connecticut and have also been described previously (Fritzell et al. 1997). Cell cultures were grown in a humidified incubator at 37°C in 5% CO_2 and were cultured in monolayer. Transformed MEFs were maintained in alpha-minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% antibiotic-antimycotic (Gibco BRL, USA). Primary MEFs (M2KO, M6KO, M2WT and M6WT), were also maintained in α-MEM supplemented with 20% fetal bovine serum and 1% antibiotic-antimycotic.

3.2 Cisplatin Treatment to Virus

The recombinant adenovirus AdCA35 (AdMCMV*lacZ*) used in these experiments was obtained from Dr. F. L. Graham at McMaster University, Hamilton ON (see Figure 1). The viral construct consists of the *Escherichia coli lacZ* reporter gene encoding β-galactosidase (β-gal) under the control of a murine cytomegalovirus early promoter

(MCMV-IE) and a SV40 polyadenylation signal inserted into the deleted early region 1 (E1) of the genome (Addison et al. 1997). Deletion of the viral E1 gene renders the virus incapable of replicating in mammalian cells except where the E1 gene is expressed in trans.

Stock cisplatin (3333 μM) used in this work was purchased as a 1 mg/mL solution (Faulding, Montreal, QC). Since cisplatin reactivity depends on the surrounding chloride ion concentration, drug concentrations were prepared by serially diluting stock cisplatin in a solution of α-MEM and low chloride Phosphate Buffered Saline (PBS) with a total chloride concentration of 50 mM. A viral suspension was then prepared in 1.8 mL of the low chloride solution in a 35 mm Petri dish (Falcon, USA) on ice. 20 μL aliquots of each cisplatin concentration were added to separate microtubes followed by the addition of 200 μL of viral suspension to each tube, producing final cisplatin concentrations of 1-8 μM. The treated virus was incubated for 12 h at 37°C after which time cisplatin damage to the viral DNA was stopped with the addition of 1 mL of unsupplemented α-MEM to each tube.

3.3 Host Cell Reactivation Assay

A schematic representation of the HCR assay is presented in Figure 1. Cells were seeded at densities ranging from $3.0x10^4$ to $4.0x10^4$ cells per well (depending on the cell line) in 96-well tissue culture plates (Falcon, Lincoln Park, NJ). Cells were then incubated for 24 h at 37°C under normoxic conditions (21% O_2) or hypoxic conditions (1% O_2). Hypoxic incubation conditions were established with a continuous flow

mixture of 94% N_2, 5% CO_2 and 1% O_2 gas. Following incubation, media was aspirated from the wells and cells were infected with 40 µL of cisplatin-treated or non-treated virus at a multiplicity of infection (MOI) ranging from 40-80 plaque forming units (pfu) per cell depending on the cell type. After 90 min of viral adsorption, excess viral solution was aspirated and supplemented growth media was added to cells. Cells were then incubated at 37°C under normoxic or hypoxic conditions for 12, 24 or 40 h and then harvested with the addition of 1 mM chlorophenolred-β-D-galactopyranoside (CPRG) (Roche, Indianapolis, IN) in 0.01% Triton X-100, 1 mM $MgCl_2$ and 100 mM phosphate buffer (pH 8.3). β-gal activity was scored using a 96-well plate reader (EL340 Bio Kinetics Reader, Bio-Tek Instruments) measuring absorbance at a wavelength of 570 nm. Saturation curves for β-gal were plotted for each cell line and only results on the linear part of the curve (prior to saturation) were used in our analyses. β-gal activity in cells was expressed relative to untreated controls (± standard error) with background levels of β-gal expression subtracted for each determinant. Each HCR experiment for a given cell line consisted of triplicate determinations for each cisplatin treatment of the virus and a minimum of three independent HCR experiments were performed for each cell line.

3.4 Clonogenic Survival Assay

Serial dilutions of cisplatin ranging from 0.5-8 µM were prepared in pre-warmed supplemented media. MMR-deficient MS5-7 cells and MMR-proficient BC1-6 cells were seeded at a density of 500 cells per well in 6-well tissue culture plates (Falcon, Lincoln Park, NJ) so as to give approximately 50-150 colonies per well. Cells were

incubated at 37°C in 5% CO_2 for a minimum of 6 h to allow for cell adhesion to the plate surface. Media was then aspirated from the wells and cells were treated with either increasing concentrations of cisplatin solution or with supplemented media (as a control). After 1 h of treatment, cisplatin solutions were aspirated, cells were rinsed in PBS and supplemented media was added to each well. Half of the plates for each cell line were then given hypoxic treatment (1% O_2) for approximately 40 h and subsequently returned to normal incubation conditions. The other plates were returned to normoxic incubation conditions only (21% O_2). After 6 days of incubation following treatment with cisplatin cells were stained with 0.5% crystal violet (Sigma, St. Louis, MO) in 70% ethanol and 10% methanol. Colonies with greater than 32 cells were scored and cell survival was expressed relative to untreated controls (± standard error). Each experiment for a given cell line consisted of triplicate determinations for each cisplatin treatment to cells and a minimum of three independent colony survival experiments were performed for each cell line.

4.0 Results

4.1 Host Cell Reactivation in MSH2-deficient and MSH2-proficient MEFs

Effects of MMR status. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA35 for MSH2-deficient and MSH2-proficient MEFs is shown in Figures 2 and 3. D_{37} is the cisplatin concentration that reduces β-gal expression to 37% and is used as a measure of HCR (see Tables II and III). Under normoxic conditions the SV40-transformed MSH2-deficient MS5-7 cells showed

significantly enhanced HCR ($P < 0.05$ by a one-sample *t*-test) relative to the MSH2-proficient BC1-6 cells. In contrast, no significant differences in repair were observed between primary MSH2-deficient M2KO cells and MSH2-proficient M2WT cells at 40h after infection. Since it was possible that primary M2KO cells had an enhanced rate of repair for cisplatin-damaged DNA compared to M2WT cells that was not detectable when β-gal activity was scored at 40 h after infection, we also measured the relative β-gal activity for the cisplatin-damaged reporter at earlier time points of 12 and 24 h following infection of the primary M2KO and M2WT cells. It can be seen that scoring for β-gal at 12 and 24 h after infection did not reveal any significant differences in repair rate between primary M2KO and M2WT cells (see Figures 4 and 5, Tables IV and V).

Effects of hypoxia. As shown in Figure 3 and Table VI, HCR of the cisplatin-damaged reporter gene in MSH2-deficient primary M2KO MEFs was significantly reduced under hypoxic (24 h pre-infection and 40 h post-infection) versus normoxic conditions when β-gal was scored at 40 h after infection. Although SV40-transformed MSH2-deficient MS5-7 cells also showed a reduced HCR under conditions of hypoxia compared to normoxia when examined at 40 h, this reduction was not significant (see Figure 2, Table VI). In contrast, MSH2-proficient primary M2WT MEFs showed a significant increase in HCR under hypoxic compared to normoxic conditions when β-gal was scored at either 40 h or 12 h after infection (see Figure 4, Table VII).

4.2 Host Cell Reactivation in MSH6-deficient and MSH6-proficient MEFs

Effects of MMR status. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA35 for primary MSH6-deficient and MSH6-proficient MEFs is shown in Figure 6. The corresponding D_{37} values are presented in Tables VIII and IX. No significant differences in HCR were observed between MSH6-deficient M6KO cells and MSH6-proficient M6WT. As shown in Figure 7 and Tables X and XI, scoring for β-gal activity at an earlier time point (24 h post-infection) also did not reveal any significant differences in repair between the two cell lines.

Effects of hypoxia. Relative D_{37} values for HCR in M6KO and M6WT cells under hypoxic (24 h pre-infection and 40 h post-infection) versus normoxic conditions are presented in Table XII. No significant effects of hypoxia were observed in either cell line when cells were scored for β-gal activity at 40 h after infection. However, when cells were scored at 24 h after infection, HCR in M6WT cells was significantly reduced under hypoxic versus normoxic conditions (see Table XIII).

4.3 Host Cell Reactivation in PMS2-deficient and PMS2-proficient MEFs

Effects of MMR status. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA35 for SV40-transformed PMS2-deficient and PMS2-proficient MEFs is shown in Figure 8. The corresponding D_{37} values are presented in Tables XIV and XV. No significant differences in repair between PMS2-deficient 334029 cells and PMS2-proficient 3340Cl 1 cells were observed.

Effects of hypoxia. Relative D_{37} values for HCR in 334029 cells and 3340Cl 1 cells under conditions of hypoxia (24 h pre-infection and 40 h post-infection) versus normoxia are presented in Table XVI. HCR in PMS2-deficient 334029 cells was significantly greater in hypoxia compared to normoxia. In contrast, no differences in repair were observed in PMS2-proficient 3340Cl 1 cells.

4.4 Survival of Cisplatin-treated SV40-transformed MEFs

Effects of MMR status. Colony survival following cisplatin treatment for SV40-transformed MSH2-deficient and MSH2-proficient MEFs are shown in Figure 9. The corresponding D_{37} values are presented in Tables XVII to XVIII. Under both normoxic and hypoxic conditions MSH2-deficient MS5-7 cells showed significantly greater survival compared to MSH2-proficient BC1-6 cells ($P < 0.05$ by a two-sample *t*-test).

Effects of hypoxia. The relative D_{37} values for survival of MS5-7 and BC1-6 cells under hypoxic versus normoxic conditions are presented in Table XIX. Survival of MSH2-deficient MS5-7 cells was significantly enhanced under hypoxic conditions compared to normoxic conditions. By contrast, no significant effect of hypoxia on the survival of MSH2-proficient BC1-6 cells was observed.

5.0 Discussion

5.1 Host Cell Reactivation in MSH2-deficient and MSH2-proficient MEFs

Effects of MMR status. We were able to demonstrate significantly greater HCR of the cisplatin-damaged reporter gene in transformed MSH2-deficient MS5-7 cells

compared to MSH2-proficient BC1-6 cells under normoxic conditions. However, we were unable to demonstrate a difference in HCR between primary MSH2-deficient M2KO cells and MSH2-proficient M2WT cells when cells were scored for β-gal at 12, 24 and 40 h after infection. These results suggest that loss of MSH2 alone does not lead to increased repair of cisplatin-damaged DNA and cellular resistance to cisplatin in MEFs. Other studies also report a weak or no association between MMR deficiency and cisplatin resistance (Claij and te Riele 2004, Sansom and Clarke 2002). These studies suggest that specific concurrent alterations in MMR-deficient cells may be necessary to confer resistance. A particularly convincing piece of evidence for this idea comes from two studies which both used MSH2-deficient and -proficient murine embryonic stem cells but showed conflicting results. One study found that the MSH2-deficient cells were significantly more resistant to cisplatin than MSH2-proficient controls (Fink et al. 1997) whereas, the other study showed no differences in the responses the MSH2-deficient and -proficient cells to cisplatin (Claij and te Riele 2004).

SV40-transformed cells have important cell-specific alterations that may contribute to resistance. Large T antigen, a protein product of simian virus 40 binds to the tumour suppressor proteins p53 and pRb preventing their interaction with other target molecules in the cell. In contrast, p53 and pRb are fully functional in primary cells. Transformed cells are also able to bypass cell cycle controls and proliferate indefinitely whereas primary cells can only be passaged a limited number of times before undergoing senescence. Studies investigating the role of MMR proteins in resistance to cisplatin have pointed to abrogated p53 as a possible candidate co-alteration required for

resistance. In fact, it has been suggested that defects in p53 may make a more important contribution to resistance than defects in MMR (Branch et al. 2000, Sansom and Clarke 2004). In the context of the current work, it is possible that the combination of SV40-transformation-induced changes (i.e. altered p53 activity) and MSH2 deficiency in MS5-7 cells result in enhanced repair of cisplatin-damaged DNA. In contrast, primary M2KO cells, which are also MSH2-deficient, may lack the requisite concomitant alterations to confer cisplatin resistance.

Effects of hypoxia. HCR was significantly reduced in primary MSH2-deficient M2KO cells under hypoxic compared to normoxic conditions. In addition, repair in SV40-transformed MSH2-deficient MS5-7 cells was also reduced in hypoxia, although this result was not significant. These findings suggest that repair of cisplatin-damaged DNA is reduced in MSH2-deficient murine cells under conditions of hypoxia. In contrast, HCR in primary MSH2-proficient M2WT cells was significantly enhanced under hypoxic conditions compared to normoxic conditions. A p53-dependent decrease in *Msh2* expression under hypoxic conditions has been reported previously (Koshiji et al. 2005). Therefore, reduction of *Msh2* expression in M2WT cells under hypoxic conditions appears to correspond with enhanced HCR. This result is surprising considering that loss of MSH2 alone did not result in repair differences between M2KO cells and M2WT cells. Complicating matters further, transformed BC1-6 cells, which are also MSH2-proficient, did not show any differences in repair under hypoxic conditions. A possible explanation for these conflicting results may be the requirement of p53 for *Msh2* downregulation in hypoxia to have a substantial impact. Koshiji et al. (2005) have

reported that p53 is required for basal levels of *Msh2* expression; therefore, in p53-proficient cells the downregulation of *Msh2* is more pronounced under hypoxic conditions. Whereas, in p53 defective cells the drop in basal MSH2 minimizes any differences in expression under hypoxic versus normoxic conditions. Since transformed BC1-6 cells have abrogated p53, differences in the expression of *Msh2* under hypoxic versus normoxic conditions may be diminished.

5.2 Host Cell Reactivation in MSH6-deficient and MSH6-proficient MEFs

Effects of MMR status. Consistent with our observations in MSH2-deficient and -proficient primary cells, we observed no differences in HCR between primary MSH6-deficient M6KO cells and MSH6-proficient M6WT cells. However, other investigators have reported increased cisplatin resistance in MSH6-defcient human colon carcinoma cells and correspondingly, increased cytotoxicity following restoration of MSH6 activity (Vaisman et al. 1998). Therefore, we conducted time-dependent experiments to determine whether scoring for β-gal activity at an earlier time point would bring out any differences in repair rate between the primary cells that were masked at 40 h post-infection. Again, no significant differences in HCR were observed between the MSH6-deficient and MSH6-proficient cells. Taken together, these findings further support the idea that loss of MMR function alone does not result in cisplatin resistance, but that additional alterations present in cells collaborate with MMR deficiency to confer resistance. Accordingly, the finding by other investigators that MSH6-deficiency significantly contributes to cisplatin resistance may have been due to the occurrence of

other incidental alterations in the human colon carcinoma cell line tested. Interestingly, Vaisman et al. (1998) have also reported that resistance in these MSH6-defcient colon carcinoma cells correlated with increased replicative bypass; therefore, enhanced replicative bypass may represent another candidate alteration that combines with MMR deficiency to produce resistance. Alternatively, continued culturing of these colon carcinoma cells may have resulted in the accumulation of other mutations necessary for resistance. Since p53 is mutated in almost half of all human cancers, this may have been one such accumulated genetic alteration that together with MSH6-deficiency leads to cisplatin resistance (Levine 1997).

Effects of hypoxia. No significant effects of hypoxia on HCR of the cisplatin-damaged reporter gene were observed in either primary MSH6-deficient M6KO cells or MSH6-proficient M6WT cells when cells were scored for β-gal activity at 40 h post-infection. However, when cells were scored for β-gal activity at 24 h post-infection a significant reduction in HCR was observed in MSH6-proficient M6WT cells under hypoxic versus normoxic conditions. It has been reported previously that expression of *Msh6* is downregulated in hypoxia (Koshiji et al. 2005). However, reduced expression of *Msh6* under hypoxic conditions did not correspond to enhanced HCR in hypoxic M6WT cells. This finding further suggests that loss of MSH6 alone cannot account for resistance to cisplatin.

5.3 Host Cell Reactivation in PMS2-deficient and PMS2-proficient MEFs

Effects of MMR status. There were no significant differences in HCR between PMS2-deficient 334029 cells and PMS2-proficient 3340Cl 1 cells. These two cell lines are SV40-transformed rendering p53 and pRb inactive; however, unlike the SV40-transformed MSH2-deficient and -proficient MEFs we tested, the combination of transformation-induced changes and MMR deficiency was not sufficient to produce differences in repair. Other investigators have reported that loss of PMS2 in immortalized MEFs results in increased cisplatin resistance (Fink et al. 1997). However, the PMS2 knockout and wildtype MEFs used in that particular study differ from the MEFs we have tested in the present work and furthermore, those cells were immortalized through serial passaging rather than SV40-transformation. It is possible that in the PMS2 knockout cells used by Fink et al. (1997), the process of immortalization may have resulted in specific cellular changes necessary for cisplatin resistance whereas, SV40-induced changes in the cells we tested were not sufficient to confer resistance. Therefore, the particular method of immortalization may also be a critical determinant of resistance. Taken together, these results suggest that loss of PMS2 alone does not lead to cisplatin resistance.

Effects of hypoxia. HCR of cisplatin-damaged DNA was significantly greater under hypoxic versus normoxic conditions in PMS2-deficient 334029 cells. These results suggest that resistance to cisplatin in the hypoxic tumour microenvironment may be a particular problem for PMS2-null tumour cells.

5.4 Survival of Cisplatin-treated SV40-transformed MEFs

Effects of MMR status. We have previously shown that HCR of the cisplatin-damaged reporter gene is significantly enhanced in MSH2-deficient MS5-7 cells relative to MSH2-proficient BC1-6 cells. In the clonogenic survival assay we have also shown significantly enhanced survival of MS5-7 cells compared to BC1-6 cells. Similar results have been reported for these cell lines in response to ionizing radiation (Fritzell et al. 1997, Peters et al. 2003). Taken together, these results suggest that elevated DNA repair contributes to cisplatin resistance in MS5-7 cells. Other reports have similarly implicated enhanced DNA repair as a key mechanism of cisplatin resistance in murine and human tumour cells (Eastman and Schulte 1988, reviewed in Chu 1994).

Effects of hypoxia. Survival of MSH2-deficient MS5-7 cells was significantly greater under hypoxic versus normoxic conditions. In contrast, HCR in these cells appears to be slightly reduced under hypoxic versus normoxic conditions. Increased genetic instability, hypermutability and decreased repair of UV-induced DNA damage have been previously reported in MMR-deficient cells exposed to hypoxic stress (Yuan et al. 2000). Taken together, these results suggest that the reduced repair we have observed in MSH2-deficient MS5-7 cells coupled with their increased survival in hypoxia may favour the accumulation of mutations resulting in genetic instability and a more aggressive tumour phenotype. We have therefore identified a possible mechanism by which genetic instability may be increased in MMR-deficient cells under hypoxic conditions.

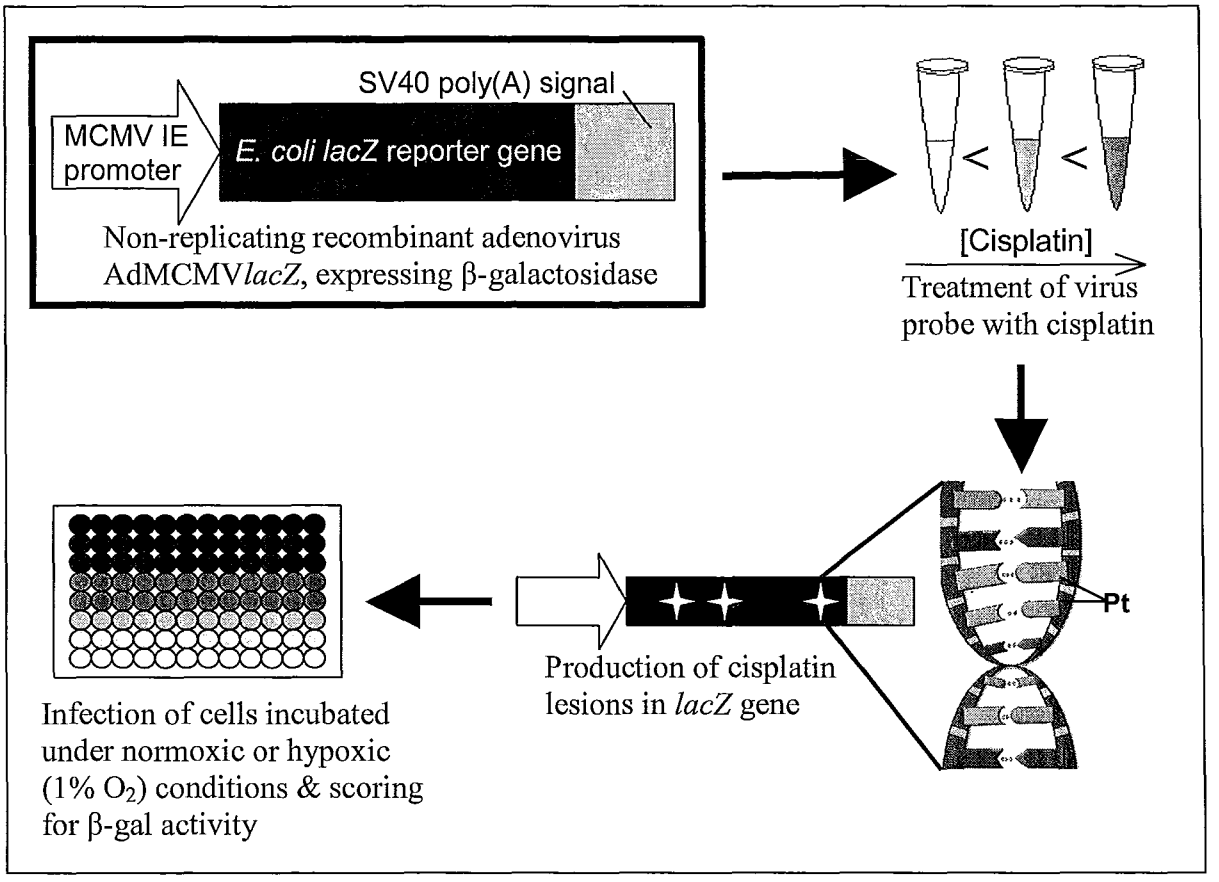

Figure 1. Schematic of the host cell reactivation assay (diagrammatic representation of the recombinant virus adapted from Addison et al. 1997; DNA image adapted from Muskopf 2007)

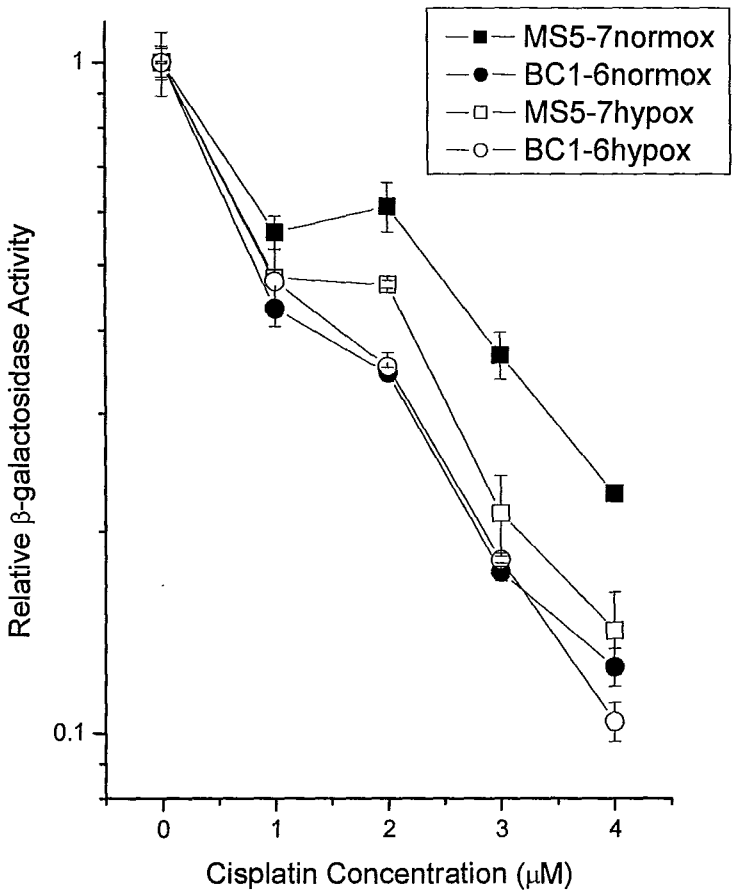

Figure 2. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in SV40-transformed MSH2-deficient MS5-7 (■,□) cells and MSH2-proficient BC1-6 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

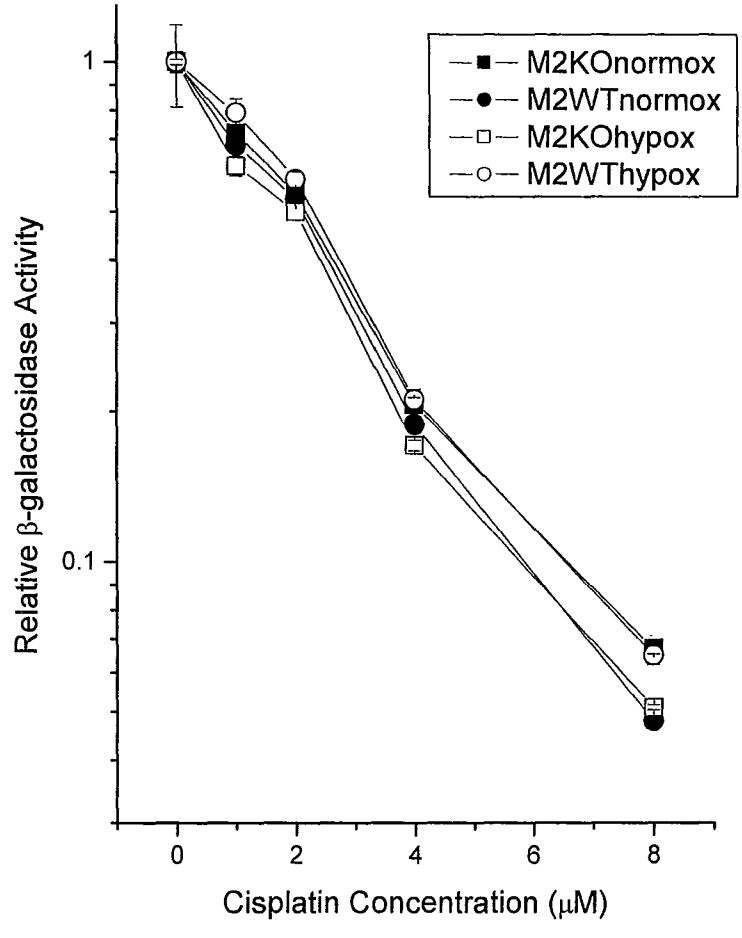

Figure 3. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in primary MSH2-deficient M2KO (■,□) cells and MSH2-proficient M2WT cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

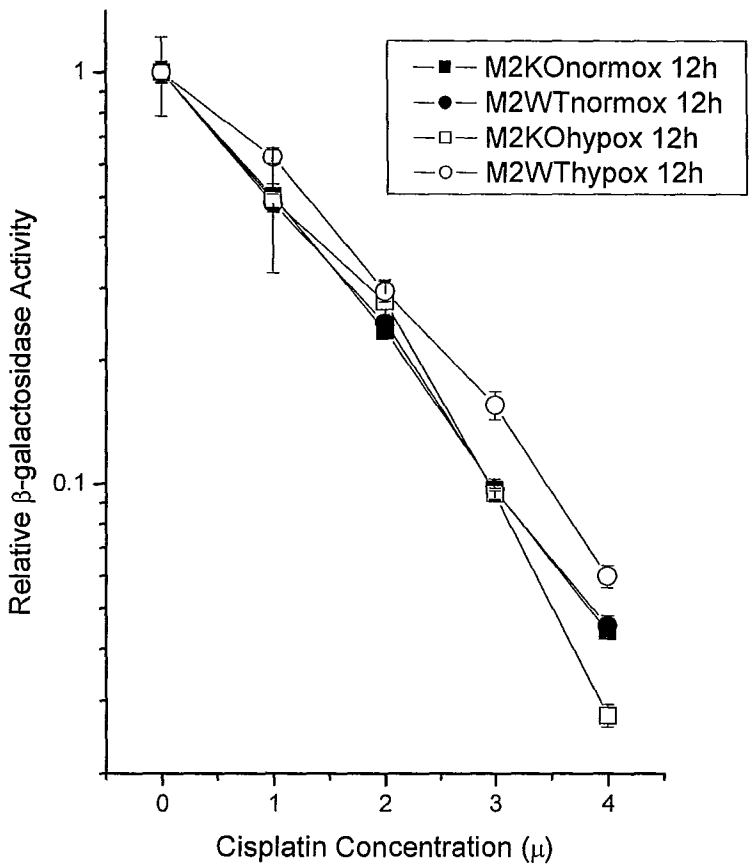

Figure 4. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in primary MSH2-deficient M2KO (■,□) cells and MSH2-proficient M2WT cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 12 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

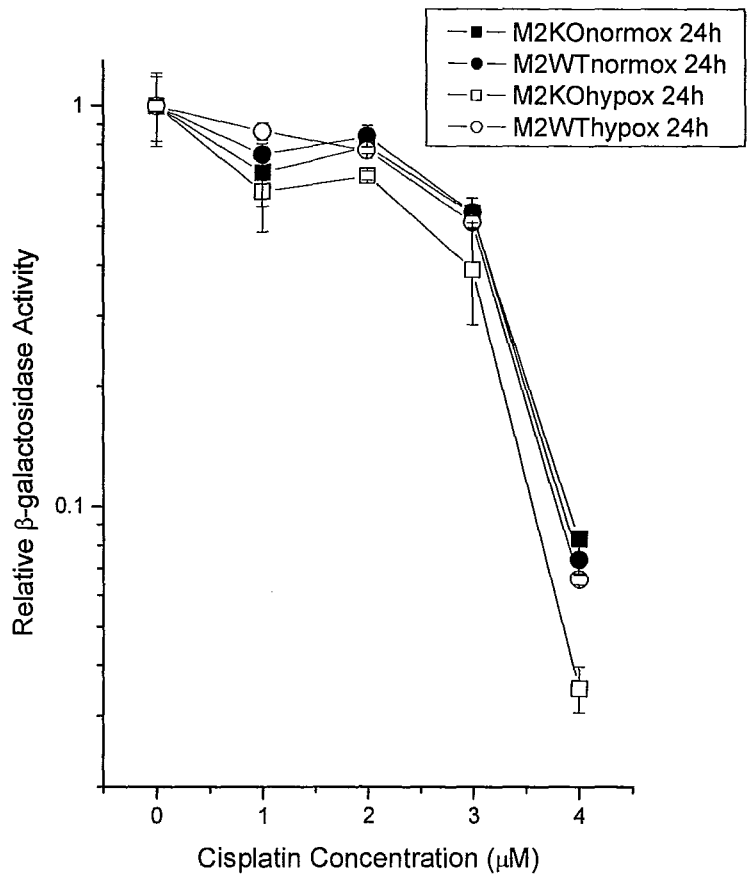

Figure 5. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in primary MSH2-deficient M2KO (■,□) cells and MSH2-proficient M2WT cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 24 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

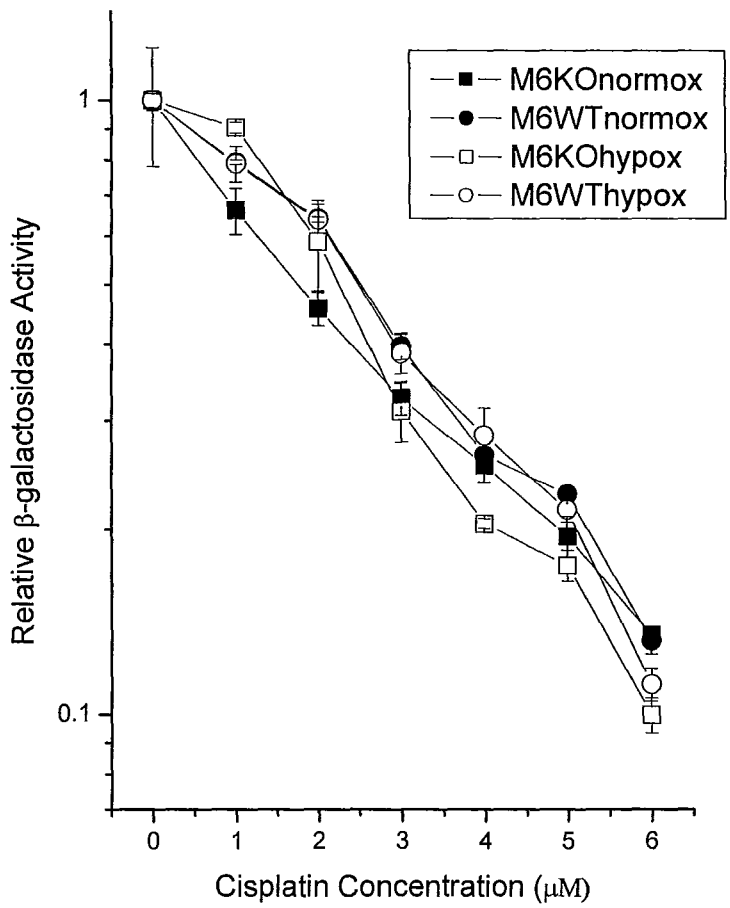

Figure 6. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in primary MSH6-deficient M6KO (■,□) cells and MSH6-proficient M6WT cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

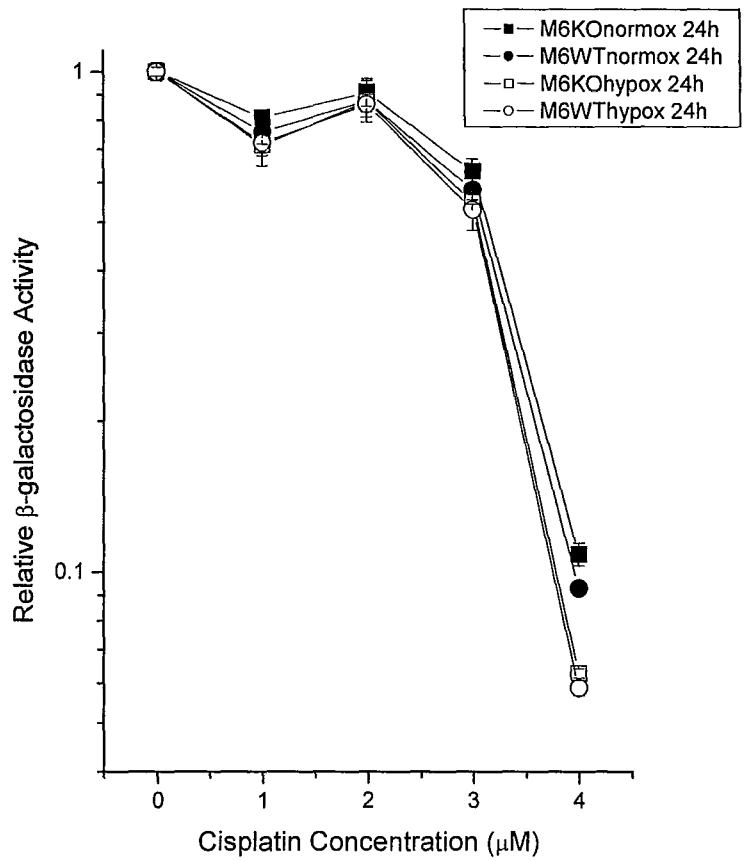

Figure 7. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in primary MSH6-deficient M6KO (■,□) cells and MSH6-proficient M6WT cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 24 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

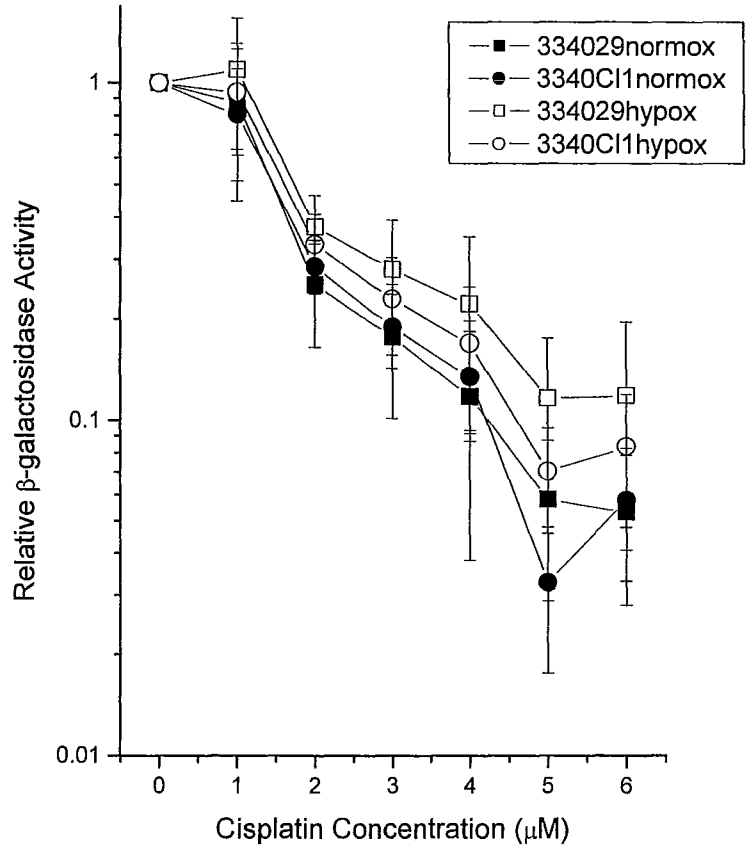

Figure 8. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in PMS2-deficient 334029 (■,□) cells and PMS2-proficient 3340Cl 1 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 5 independent experiments each performed in triplicate.

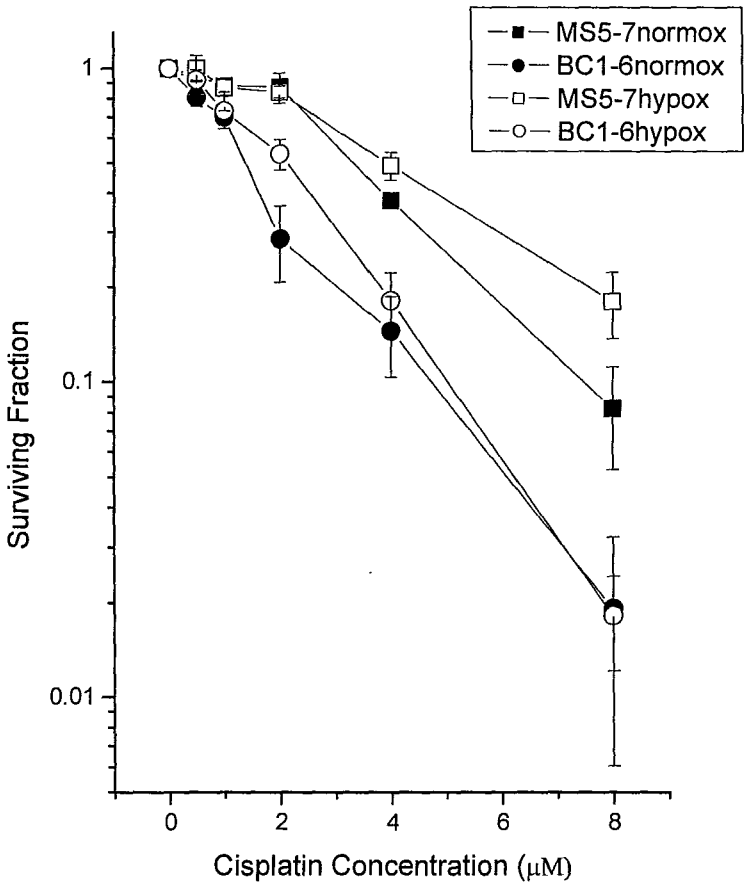

Figure 9. Clonogenic survival of cisplatin-treated SV40-transformed MSH2-deficient MS5-7 (■,□) cells and MSH2-proficient BC1-6 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and treated 6 h later with serial dilutions of cisplatin or supplemented media for 1 h. Cells were returned to normoxic incubation for 6 days or were incubated in hypoxia for 40 h and returned to normoxic incubation for 4 days. Cells were then stained and scored for colony formation. Each point represents the average ± SE of 3 independent experiments each performed in triplicate.

Table I. Murine Embryonic Fibroblasts used

Cell Line	Type	MMR protein	MMR +/-	Primary/ transformed	Other Information
M2KO	Murine	MSH2	-	Primary	Functional p53
M2WT	Murine	MSH2	+	Primary	Functional p53
MS5-7	Murine	MSH2	-	SV40-transformed	Abrogated p53
BCl-6	Murine	MSH2	+	SV40-transformed	Abrogated p53
M6KO	Murine	MSH6	-	Primary	Functional p53
M6WT	Murine	MSH6	+	Primary	Functional p53
334029	Murine	PMS2	-	SV40-transformed	Abrogated p53
3340Cl1	Murine	PMS2	+	SV40-transformed	Abrogated p53

Table II. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH2-deficient and MSH2-proficient MEFs

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
M2KO (MSH2-)	5	3.61±0.69	3.37±0.66
M2WT (MSH2+)	5	3.55±0.46	3.96±0.52
MS5-7 (MSH2-)	5	4.29±1.01	3.76±0.74
BC1-6 (MSH2+)	5	3.07±0.65	3.25±0.53

Table III. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH2-deficient versus MSH2-proficient MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M2KO/M2WT (Normoxia)	5	0.977±0.069	0.97
MS5-7/BC1-6 (Normoxia)	5	1.41±0.12	**0.027**[a]
M2KO/M2WT (Hypoxia)	5	0.840±0.076	0.10
MS5-7/BC1-6 (Hypoxia)	5	1.15±0.06	0.071

[a]significantly >1 by a one-sample *t*-test (P<0.05)

Table IV. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 12 and 24 h post-infection in MSH2-deficient and MSH2-proficient primary MEFs

Cell Lines	No.	D_{37} Normoxia ± SE 12 h (μM)	D_{37} Hypoxia ± SE 12 h (μM)	D_{37} Normoxia ± SE 24 h (μM)	D_{37} Hypoxia ± SE 24 h (μM)
M2KO	3	1.98±0.52	2.15±0.36	2.41±0.48	2.08±0.61
M2WT	3	2.18±0.40	2.54±0.42	2.59±0.56	2.94±0.50

Table V. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 12 and 24 h post-infection in MSH2-deficient versus MSH2-proficient primary MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M2KO/M2WT (Normoxia 12 h)	3	0.912±0.149	0.62
M2KO/M2WT (Hypoxia 12 h)	3	0.848±0.046	0.080
M2KO/M2WT (Normoxia 24 h)	3	0.953±0.082	0.62
M2KO/M2WT (Hypoxia 24 h)	3	0.682±0.144	0.16

Table VI. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH2-deficient and MSH2-proficient MEFs under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M2KO Hypoxia/Normoxia	5	0.932±0.013	**0.0063**[a]
M2WT Hypoxia/Normoxia	5	1.12±0.04	**0.046**[b]
MS5-7 Hypoxia/Normoxia	5	0.899±0.048	0.10
BC1-6 Hypoxia/Normoxia	5	1.09±0.05	0.14

[a]significantly <1 by a one-sample *t*-test (P<0.05)
[b]significantly >1 by a one-sample *t*-test (P<0.05)

Table VII. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 12 and 24 h post-infection in MSH2-deficient and MSH2-proficient primary MEFs under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M2KO Hypoxia/Normoxia (12 h)	3	1.14±0.16	0.46
M2WT Hypoxia/Normoxia (12 h)	3	1.17±0.03	**0.029**[a]
M2KO Hypoxia/Normoxia (24 h)	3	0.823±0.111	0.25
M2WT Hypoxia/Normoxia (24 h)	3	1.18±0.11	0.24

[a]significantly >1 by a one-sample *t*-test (P<0.05)

Table VIII. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH6-deficient and MSH6-proficient primary MEFs

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
M6KO (MSH6-)	3	1.52±0.60	1.51±0.66
M6WT (MSH6+)	3	1.72±0.73	1.85±0.66

Table IX. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH6-deficient versus MSH6-proficient primary MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M6KO/M6WT (Normoxia)	3	0.888±0.145	0.52
M6KO/M6WT (Hypoxia)	3	0.759±0.138	0.22

Table X. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 24 h post-infection in MSH6-deficient and MSH6-proficient primary MEFs

Cell Lines	No.	D_{37} Normoxia ± SE 24 h (μM)	D_{37} Hypoxia ± SE 24 h (μM)
M6KO	3	2.52±0.63	2.26±0.51
M6WT	3	2.50±0.50	2.41±0.49

Table XI. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 24 h post-infection in MSH6-deficient versus MSH6-proficient primary MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M6KO/M6WT (Normoxia 24 h)	3	0.979±0.087	0.83
M6KO/M6WT (Hypoxia 24 h)	3	0.932±0.047	0.29

Table XII. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h post-infection in MSH6-deficient and MSH6-proficient primary MEFs under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M6KO Hypoxia/Normoxia	3	0.953±0.065	0.54
M6WT Hypoxia/Normoxia	3	1.13±0.08	0.23

Table XIII. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 24 h post-infection in MSH6-deficient and MSH6-proficient primary MEFs under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
M6KO Hypoxia/Normoxia (24 h)	3	0.937±0.110	0.62
M6WT Hypoxia/ Normoxia (24 h)	3	0.964±0.005	**0.022**[a]

[a]significantly <1 by a one-sample *t*-test ($P<0.05$)

Table XIV. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h in PMS2-deficient and PMS2-proficient MEFs

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
334029 (PMS2-)	5	1.84±0.63	2.42±0.68
3340Cl 1 (PMS2+)	5	1.61±0.25	2.32±0.59

Table XV. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h in PMS2-deficient versus PMS2-proficient primary MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
334029/3340Cl 1 (Normoxia)	5	1.09±0.28	0.77
334029/3340Cl 1 (Hypoxia)	5	1.03±0.06	0.58

Table XVI. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* at 40 h in PMS2-deficient and PMS2-proficient primary MEFs under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
334029 Hypoxia/Normoxia	5	1.44±0.14	**0.035**[a]
3340Cl 1 Hypoxia/Normoxia	5	1.41±0.22	0.14

[a]significantly >1 by a one-sample *t*-test ($P<0.05$)

Table XVII. Average D_{37} values from clonogenic survival of cisplatin-treated MSH2-deficient and MSH2-proficient transformed MEFs

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
MS5-7 (MSH2-)	3	4.06±0.09	4.99±0.08
BC1-6 (MSH2+)	3	1.81±0.29	2.67±0.28

Table XVIII. MMR Effects: Relative D_{37} values from clonogenic survival of cisplatin-treated MSH2-deficient versus MSH2-proficient transformed MEFs

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
MS5-7/BC1-6 (Normoxia)	3	2.37±0.41	**0.0018**[a]
MS5-7/BC1-6 (Hypoxia)	3	1.90±0.16	**0.0014**[a]

[a]significantly >1 by two-sample *t*-test (P<0.05)

Table XIX. Hypoxia Effects: Relative D_{37} values from clonogenic survival of cisplatin-treated MSH2-deficient and MSH2-proficient transformed MEFs under normoxic versus hypoxic conditions

Cell Lines	No.	Avg. D37 Ratio ± SE	P Value
MS5-7 Hypoxia/Normoxia	3	1.23±0.025	**0.0017**[a]
BC1-6 Hypoxia/Normoxia	3	1.57±0.31	0.10

[a]significantly >1 by two-sample *t*-test (P<0.05)

CHAPTER 3

Repair of Cisplatin-damaged DNA in p53 Compromised, Mismatch Repair-Deficient and Normal Human Cells

1.0 Abstract

Much of the research on the underlying mechanisms of resistance to cisplatin has focused on the mismatch repair (MMR) system. Loss of several MMR proteins has been implicated in intrinsic and acquired cisplatin resistance in tumour cells (Aebi et al. 1996, Cenni et al. 1999, Vaisman et al. 1998). However, we and others have shown previously that loss of MMR function alone may not be able to account for resistance in some cell lines and that other concomitant alterations may be required (Chapter 2, Claij and te Riele 2004, Sansom and Clarke 2002). Such alterations may include transformation-induced changes and/or loss of p53. The hypoxic tumour microenvironment which results from an imbalance in oxygen supply and tissue demand has also been shown to affect cell responses to cancer treatments. We have examined the effects of p53 status, SV40-transformation, MMR status and hypoxia on host cell reactivation (HCR) of a cisplatin-damaged *lacZ* reporter gene encoded by a recombinant virus in a panel of human Li-Fraumeni Syndrome (LFS) cells, normal cells and tumour cells. No significant differences in HCR were observed between immortalized (p53-/-) and primary (p53+/-) LFS cells. Similarly, no differences in HCR were observed between immortalized LFS cells (p53-/-) and human normal fibroblasts with wildtype p53 (+/+). In human normal cells, greater HCR was observed in SV40-transformed GM637F cells relative to primary normal fibroblasts. Additionally, hypoxic treatment 24 h pre-infection and 40 h post-infection reduced HCR in GM637F cells but had no effect on repair in the primary cell strains. HCR in hMLH1-deficient HCT116 colon carcinoma cells was significantly enhanced relative to isogenic HCT116 hMLH1-proficient controls consistent with a role

for hMLH1 in reduced repair of cisplatin-damaged DNA. In contrast, HCR in hMSH2-deficient LoVo cells was significantly reduced compared to hMSH2-proficient SW480 cells indicating that cellular alterations in tumour cells other than MMR deficiency can also contribute to repair of cisplatin-damaged DNA. Hypoxia 24 h pre-infection and 40 h post-infection significantly reduced HCR in three out of the six human tumour cell lines we examined including an hMLH1-deficient line and two MMR-proficient cell lines. Our findings indicate that effects of hypoxia on repair of cisplatin-damaged DNA vary with cell type and are not limited to human tumour cells with MMR-deficiencies. Taken together, these results suggest that cell changes associated with SV40-transformation confer a repair advantage for cisplatin-damaged DNA compared to primary cell strains. Our results from the human tumour lines examined indicate that loss of hMLH1 alone may be sufficient for enhanced repair of cisplatin damage in some tumour cells confirming a previous report (Cenni et al. 1999), but hMSH2 loss may not, in contrast to other findings (Cenni et al. 1999, Fink et al. 1996). Our results also suggest that hypoxic effects on the repair of cisplatin-damaged DNA vary with cell type and hypoxia may specifically reduce repair in SV40-transformed cells. Reduced HCR of a UV-damaged reporter gene has similarly been reported in a transformed mouse cell line and a human colorectal carcinoma cell line under hypoxic conditions (Yuan et al. 1999; Dregoesc 2007, unpublished results).

2.0 Introduction

Tumour cell resistance to the platinum-based chemotherapy drug cisplatin remains an elusive problem for clinicians and is the most common reason for treatment failure. Defects in the mismatch repair (MMR) system have been linked to cisplatin resistance as well as hereditary forms of colorectal cancer and some endometrial and ovarian cancers (as reviewed in Fishel and Kolodner 1995). Therefore, clinical treatment of these tumour types poses a particular problem since they often show intrinsic resistance to cisplatin. The tumour microenvironment characterized by hypoxia, low pH and nutrient deprivation also influences cell responses to DNA damaging agents. Alterations in the expression of DNA repair genes, decreases in DNA repair and increased genetic instability have all been reported in hypoxic cells (Mihaylova et al. 2003, Koshiji et al. 2005, Yuan et al. 2000). However, the effects of hypoxia on the repair of cisplatin-damaged DNA in cells have not been characterized and warrant further examination.

Resistance to cisplatin has been demonstrated *in vitro* and *in vivo* in both human adenocarcinoma cells and murine cells with defects in MHL1, MSH2, MSH6 and PMS2 (Abei et al. 1996, Cenni et al. 1999, Fink et al. 1996, Fink et al. 1997 and Vaisman et al. 1998). However, reports suggesting that loss of MMR alone cannot account for resistance are accumulating (Claij and te Riele 2004, Sansom and Clarke 2002). We have also reported that loss of MMR by itself cannot account for cisplatin resistance in murine embryonic fibroblasts (MEFs) with defects in MSH2, MSH6 and PMS2 (Chapter 2). Of particular interest was our finding that resistance depended on the transformation status

of the cell. Specifically, we found that SV40-transformed MEFs deficient in MSH2 demonstrated increased HCR of a cisplatin-damaged reporter gene relative to wildtype controls; however, no differences in repair were observed between primary MSH2-deficient and -proficient MEFs. These results suggested that transformation-induced changes or other acquired genetic alterations in cells may collaborate with MMR deficiency to confer cisplatin resistance. Similarly, several studies have suggested that additional cell-specific alterations may be requisite in MMR-deficient cells to confer resistance (Branch et al. 2000, Claij and te Riele 2004, Helleman et al. 2006, Sansom and Clarke 2002).

Several lines of evidence from our own studies and those of other investigators have pointed to defective p53 as a possible alteration required for resistance in MMR-deficient cells. The *p53* tumour suppressor gene is mutated in as many as 50% of all human cancers (as reviewed in Levine 1997). Under normal conditions, endogenous p53 levels are low due to binding of mdm2 with p53, which targets the protein for degradation in an autoregulatory loop (as reviewed in Ahuja et al. 2005). However, various cellular stressors such as DNA damage, hypoxia, pH changes and heat shock result in stabilization of p53 through inhibition of its interaction with mdm2 (as reviewed in Ashcroft and Vousden 1999, Meek 2004). Stabilization of the p53 protein appears to involve a diversity of responses including phosphorylation of p53, transcriptional downregulation of *Mdm2* and post-translational modification of mdm2. In its activated state, p53 may upregulate the expression of several genes involved in cell cycle control, DNA repair and apoptotic signaling. Consequently, cell cycle arrest is activated allowing

for DNA repair to take place or alternatively, cells undergo apoptosis if the damage is too extensive. In addition to upregulating effectors of DNA repair, p53 may also directly participate in DNA repair activities as indicated through its binding to primary DNA damage (Degtyareva et al. 2001, Lee et al. 1995, Reed et al. 1995). SV40-transformation of cells inhibits the activity of p53 (as well as pRb proteins), since large T antigen, a protein product of SV40 binds to p53 preventing its transcriptional activation of downstream gene targets. However, whether this interaction is sufficient to fully suppress all functions of p53 is controversial as some studies have found large amounts of unbound p53 in SV40-transformed cells and other studies have demonstrated some p53 activity in SV40-transformed cells (Hess and Brandner 1997, O'Neill et al. 1997). Notwithstanding, our finding that SV40-transformed MSH2-deficient murine cells but not primary MSH2-deficient cells demonstrated cisplatin resistance suggests that abrogated p53 and/or abrogation of the pRb family of proteins may contribute to resistance.

The literature on the functional consequences of p53 inactivation in tumour cells is conflicting with some studies showing that p53 loss increases cell sensitivity to cisplatin (Fan et al. 1995, Fedier et al. 2002, Hawkins et al. 1996, Pestell et al. 2000, Vikhanskaya et al. 1999) whereas, other reports indicate that resistance to cisplatin is increased (Branch et al. 2000, Fujiwara et al. 1994, Kondo et al. 1995, Sansom and Clarke 2002). With respect to the clinical consequences of p53 defects, Kigawa et al. (2001) have reported that 83% of non-responding patients undergoing cisplatin therapy for ovarian cancer had a mutation in the *p53* gene compared to only 16% of responders.

In that study, apoptosis was also shown to be greater in tumours expressing wildtype p53 and restoration of p53 activity in p53-deficient tumours sensitized them to cisplatin. Critically, in some cell lines p53 and MMR defects have been shown to act synergistically in conferring resistance to cisplatin. Lin and Howell (2006) have reported that loss of both MMR and p53 function increases the rate at which cisplatin resistant variants emerge in a population of tumour cells during subsequent rounds of cisplatin treatment. Some reports have suggested that p53 loss may be a more important determinant of cisplatin resistance than MMR deficiency in some cell lines. For example, Branch et al. (2000) have reported that introduction of a hMLH1 defect into ovarian cancer cells resulted in only a slight increase in survival whereas, introduction of a p53 defect significantly increased cisplatin resistance. Likewise, Sansom and Clarke (2002) reported that p53 deficiency but not MSH2 deficiency led to increased survival in murine intestinal cells following cisplatin treatment.

In the present work we have investigated the effects of p53 deficiency and hypoxia on host cell reactivation (HCR) of a cisplatin-damaged reporter gene in Li-Fraumeni Syndrome (LFS) cells with heterozygous and homozygous defects in the *p53* gene. Germline mutations in *p53* are characteristic of LFS and while some tumours in affected individuals remain heterozygous for *p53* the development of other tumours requires a "second hit" to the remaining wildtype allele. Individuals with LFS are predisposed to developing various cancers at an early age including breast cancer, soft tissue sarcomas, brain tumours, osteosarcomas and leukemia (as reviewed in Malkin 1993). Affected individuals are also prone to developing multiple primary tumours.

We have extended our investigation of the effects of p53 and hypoxia on repair of cisplatin-damaged DNA to normal human fibroblasts with abrogated or wildtype p53 (SV40-transformed and primary cells respectively). While mutated or abrogated p53 represent likely candidate alterations required for chemoresistance in MMR-deficient cells, it is also possible that other cell-specific changes induced by SV40-transformation such as abrogated pRb or alterations in other regulatory molecules may be necessary for resistance. Use of mutant p53-expressing LFS cells, SV40-transformed cells and primary human normal controls will facilitate a determination of whether p53 mutation, p53 abrogation and/or transformation-induced alterations or none of the above contribute to resistance in MMR-deficient cells. We have used more than one primary normal cell strain in these studies to establish whether any observed differences in repair may be due to peculiarities in one or more of the primary lines.

The use of normal fibroblasts in the present work also serves as an important comparison for tumour-derived cells, allowing us to determine how cells normally respond to cisplatin chemotherapy under both standard and hypoxic conditions. The identification of key differences in repair between normal and tumour cells may help to pinpoint specific genetic alterations in tumour cells that may be important in determining responses to chemotherapy (for example, factors mediating hypoxic effects). Information on how cisplatin damage is repaired in normal tissues may also be useful in understanding toxic side effects given that cisplatin diffuses into both healthy and tumourgenic tissues.

Finally we have examined HCR of cisplatin-damaged DNA under normoxic and hypoxic conditions in a panel of MMR-deficient and -proficient human adenocarcinoma cells. We sought to determine whether the responses of these cells to cisplatin could be predicted given an understanding of the effects of MMR deficiency, p53 mutation, abrogated p53, SV40-transformation and hypoxia on repair of cisplatin-damaged DNA. Few studies have examined repair of cisplatin-damaged DNA in the context of a HCR assay in MMR-deficient and -proficient cells. In addition, we are not aware of any previous studies on the effects of hypoxia on repair of cisplatin-damaged DNA in tumour cells.

3.0 Materials and Methods

3.1 Cell Lines

Human LFS cells, normal fibroblasts and adenocarcinoma cells are described in Table I. Immortalized and primary LFS087 cells were kindly provided by Dr. M.A. Tainsky and B.A. Darmanos at Wayne State University Cancer Institute, Detroit, MI and have been described previously (Yin et al. 1992). hMLH1-proficient HCT116+ch3 and hMLH1-deficient HCT116 human colon adenocarcinoma cells were kindly provided by Dr. T. Kunkel at the National Institute of Environmental Health Sciences, Research Triangle Park, NC and have been described previously (Koi et al. 1994). All human normal fibroblasts including SV40-transformed GM637F cells and primary GM9503, GM969C and GM8399 skin fibroblasts were purchased from the National Institute of General Medical Sciences, Camden, NJ. MMR-proficient SW480 and MSH2-deficient

LoVo human colon carcinoma cells were purchased from the American Type Culture Collection. hPMS2-deficient HEC1A human endometrial carcinoma cells and hMLH1- and hPMS2-deficient SW48 human colon carcinoma cells were generously provided by Dr. A. Guarné and V. Leong at McMaster University, Hamilton, ON. Cell cultures were grown in a humidified incubator at 37°C in 5% CO_2 and were cultured in monolayer. All cells were maintained in alpha-minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% antibiotic-antimycotic (Gibco BRL, USA).

3.2 Cisplatin Treatment to Virus

The recombinant adenoviruses AdCA17 (AdHCMV*lacZ*) and AdCA35 (AdMCMV*lacZ*) used in these experiments were obtained from Dr. F. L. Graham at McMaster University, Hamilton ON. Both viral constructs consist of an *E. coli lacZ* reporter gene encoding β-galactosidase (β-gal) under the control of a murine or human cytomegalovirus early promoter (MCMV-IE and HCMV-IE respectively) and a SV40 polyadenylation signal (Addison et al. 1997). All three components have been inserted into the deleted early region 1 (E1) of the viral genome. Deletion of the viral E1 gene renders the virus incapable of replicating in mammalian cells except where the E1 gene is expressed in trans.

Stock cisplatin (3333 μM) used in this work was purchased as a 1 mg/mL solution (Faulding, Montreal, QC). Stock cisplatin was serially diluted in a solution of α-MEM and low chloride PBS (50 mM Cl^-) to activate the drug. A viral suspension was then

prepared in 1.8 mL of the low chloride solution in a 35 mm diameter Petri dish (Falcon, USA) on ice. 20 μL aliquots of each cisplatin concentration were added to separate microtubes followed by the addition of 200 μL of viral suspension to each tube, producing final cisplatin concentrations of 1-6 μM. The treated virus was incubated for 12 h at 37°C after which time cisplatin damage to the viral DNA was stopped with the addition of 1 mL of unsupplemented α-MEM to each tube.

3.3 Host Cell Reactivation Assay

Cells were seeded at densities ranging from $2.0x10^4$ to $3.5x10^4$ cells per well (depending on the cell line) in 96-well tissue culture plates (Falcon, Lincoln Park, NJ). Cells were then incubated for 24 h at 37°C under normoxic conditions (21% O_2) or hypoxic conditions (1% O_2 as described in Chapter 2). Following incubation, media was aspirated from the wells and cells were infected with 40 μL of cisplatin-treated or non-treated virus at a multiplicity of infection (MOI) ranging from 40-190 plaque forming units (pfu) per cell depending on the cell type. After 90 min of viral adsorption, excess viral solution was aspirated and supplemented growth media was added to the cells. Cells were then incubated at 37°C under normoxic or hypoxic conditions for 40 h and then harvested with the addition of 1 mM CPRG (Roche, Indianapolis, IN) in 0.01% Triton X-100, 1 mM $MgCl_2$ and 100 mM phosphate buffer (pH 8.3). β-gal activity was scored using a 96-well plate reader (EL340 Bio Kinetics Reader, Bio-Tek Instruments) measuring absorbance at a wavelength of 570 nm. β-gal activity in cells was expressed relative to untreated controls (± standard error) as described in Chapter 2. Each HCR

experiment for a given cell line consisted of triplicate determinations for each cisplatin treatment of the virus and a minimum of three independent HCR experiments were performed for each cell line.

4.0 Results

4.1 Host Cell Reactivation in Mutant p53 Expressing Li-Fraumeni Syndrome Cells and Normal Human Fibroblasts

Effects of p53 mutation. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA17 for LFS087 immortalized ($LFS^{p53-/-}$) and primary cells ($LFS^{p53+/-}$) is shown in Figure 1. D_{37} is the cisplatin concentration that reduces the expression of β-gal activity to 37% and is used as a measure of HCR (see Tables II and III). No significant differences in HCR were observed between $LFS^{p53-/-}$ cells and $LFS^{p53+/-}$ cells.

Examination of HCR in $LFS^{p53-/-}$ cells compared to SV40-transformed GM637F normal human cells also did not reveal any significant differences (see Fig. 2 and Table IV). Similarly, no significant differences in HCR were observed between $LFS^{p53-/-}$ cells and primary GM9503 human normal fibroblasts.

Effects of hypoxia. Relative D_{37} values for HCR in $LFS^{p53-/-}$ cells and $LFS^{p53+/-}$ cells under hypoxic (24 h pre-infection and 40 h post-infection) versus normoxic conditions are presented in Table V. No significant effects of hypoxia were observed in either of the LFS cell lines.

4.2 Host Cell Reactivation in SV40-transformed and Primary Human Normal Fibroblasts

Effects of SV40-transformation. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA17 for SV40-transformed and primary human normal fibroblasts is shown in Figures 3 to 5 and the corresponding D_{37} values are presented in Tables VI and VII. Under normoxic conditions SV40-transformed GM637F cells showed significantly enhanced HCR ($P < 0.05$) relative to primary normal fibroblasts when results were pooled. This enhancement of HCR in SV40-transformed cells compared to primary cells was similar for GM9503, GM8399 and GM969C cells with relative HCR values of 1.27, 1.48 and 1.44 respectively.

Effects of hypoxia. Relative D_{37} values for HCR in all normal cell lines under hypoxic (24 h pre-infection and 40 h post-infection) versus normoxic conditions are presented in Table VIII. HCR of the cisplatin-damaged reporter gene in SV40-transformed GM637F cells was significantly reduced under hypoxic compared to normoxic conditions. In contrast, no significant effects of hypoxia were observed in any of the primary cell lines individually or when results for these cells were pooled.

4.3 Host Cell Reactivation in MMR-deficient and MMR-proficient Human Adenocarcinoma Cells

Effects of MMR status. The relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA17 for MMR-deficient and -proficient human adenocarcinoma cells is shown in Figures 6 to 8 and the corresponding D_{37} values are presented in Tables IX and X. Under normoxic conditions HCR was significantly

enhanced in hMLH1-deficient HCT116 cells compared to hMLH1-proficient HCT116+ch3 cells. In contrast, HCR in hMSH2-deficient LoVo cells was significantly reduced relative to hMSH2-proficient SW480 cells in both incubation conditions.

Effects of hypoxia. Relative D_{37} values for all adenocarcinoma cell lines examined under hypoxic (24 h pre-infection and 40 h post-infection) versus normoxic conditions are presented in Table XI. HCR in hMLH1-deficient HCT116 cells, hMLH1-proficient HCT116+ch3 cells and hMSH2-proficient SW480 cells was significantly reduced under hypoxic versus normoxic conditions. In contrast, no significant effects of hypoxia on HCR were observed in hPMS2-deficient HEC1A cells, hMSH2-deficient LoVo cells or in hMLH1- and hPMS2-deficient SW48 cells.

HCR of the AdCA35lacZ virus. The AdCA35*lacZ* virus containing a murine CMV promoter was also used in HCT116, HCT116+ch3, LoVo and SW480 colon carcinoma cells to facilitate increased expression of the *lacZ* reporter gene. Relative β-gal activity of the cisplatin-damaged reporter gene at 40 h after infection with AdCA35 for these cell lines is shown in Figures 9 and 10. The corresponding D_{37} values are presented in Tables XII to XIV. In contrast to the results obtained with the AdCA17*lacZ* viral construct (described above), no significant differences in repair were observed between hMLH1-deficient HCT116 cells and hMLH1-proficient HCT116+ch3 cells. Additionally, under normoxic conditions no significant differences were observed between hMSH2-deficient LoVo cells and hMSH2-proficient SW480 cells; however, under hypoxic conditions HCR was significantly reduced in LoVo cells compared to SW480 cells.

Hypoxic treatment for 24 h pre-infection and 40 h post-infection had no significant effects on HCR in HCT116, HCT116+ch3, LoVo or SW480 cells.

5.0 Discussion

5.1 Host Cell Reactivation in Mutant p53 Expressing Li-Fraumeni Syndrome Cells and Normal Human Fibroblasts

Effects of p53 mutation. Ford and Hanawalt (1995) have reported decreased global genome repair of UVC-induced cyclobutane pyrimidine dimers (CPDs) in $LFS^{p53-/-}$ cells but not in $LFS^{p53+/-}$ cells; however, they also found that transcription-coupled repair was near normal in $LFS^{p53-/-}$ cells (demonstrated by a similar rate and extent of repair of transcribed genes as in human normal fibroblasts). In contrast, other studies suggest some involvement of p53 in transcription-coupled repair of UV-induced CPDs, although this involvement remains controversial (Dregoesc et al. 2006, Mathonnet et al. 2003, Mathonnet et al. 2004, McKay et al. 2001, Therrien et al. 1999). Specifically, Dregoesc et al. (2006) have reported that increased expression of p53 leads to increased TCR and GGR of a UVC-damaged reporter gene in human fibroblasts. McKay et al. (2001) have reported that disruption of p53 in TCR-proficient cells increased their sensitivity to both UV-induced and cisplatin-induced apoptosis. Removal of UVB-induced CPDs from both transcribed and non-transcribed strands of active genes has been shown to be defective in p53-deficient cells (Therrien et al. 1999). Additionally, previous work in our lab (Davis 1996), has shown that cisplatin-treated p53-heterozygous LFS cells have a reduced capacity to support viral DNA synthesis compared to normal

controls. This capacity assay correlates closely with TCR of cellular DNA (McKay et al. 1997) and suggests that p53 deficiency reduces TCR of cisplatin-induced DNA damage in human cells.

In the present report we examined repair of the cisplatin-damaged reporter gene in $LFS^{p53-/-}$ cells and in $LFS^{p53+/-}$ cells for comparison. We did not find any significant differences in HCR between $LFS^{p53-/-}$ cells and $LFS^{p53+/-}$ cells. This result suggests that p53 mutant and p53-heterozygous LFS cells may be functionally equivalent in terms of repair capacity for cisplatin-damaged DNA. This may be due to insufficient expression of wildtype p53 in p53-heterozygous LFS cells, no involvement of p53 in HCR of the cisplatin-damaged reporter gene or a dominant negative effect of the mutated p53 protein on wildtype p53 function in $LFS^{p53+/-}$ cells.

Examination of HCR in $LFS^{p53-/-}$ cells and p53-proficient GM9503 normal cells also did not reveal any significant differences in HCR. However, it is noteworthy that on average repair in GM9503 cells was higher relative to repair in $LFS^{p53-/-}$ cells under normoxic conditions and this difference approached significance ($P = 0.07$). Additional experiments may reveal this to be a significant effect. If significant, this would indicate an involvement of p53 in the repair of cisplatin-damaged DNA and would lend further support to reports suggesting that defects in p53 may sensitize cells to cisplatin (Fan et al. 1995, Hawkins et al. 1996, Pestell et al. 2000). However, it remains to be determined whether the possible role of p53 in the repair of cisplatin damage may reflect an involvement in the GGR and/or TCR subpathways of NER or in the homologous recombination repair pathway. Given the role of p53 in regulating cell cycle arrest and

transcription of DNA repair genes, it seems plausible that p53 deficiency in cells would result in reduced repair.

Similarly, no significant differences in HCR were observed between $LFS^{p53-/-}$ cells and SV40-transformed GM637F cells with abrogated p53. This result suggests that the effect of abrogated p53 in GM637F cells due to binding with large T antigen may be functionally equivalent to a homozygous mutation of p53 as in immortalized $LFS^{p53-/-}$ cells. It is worth noting however, that the effect of mutant p53 in cells may be very different to the effect of complete p53 loss. Additionally, the results for GM637F cells do not rule out the possibility that these cells may retain some p53 activity, although it is not sufficient to produce a statistically significant enhancement of HCR.

Taken together, these results suggest that p53 mutation alone does not result in increased repair of cisplatin-damaged DNA. However, the mechanisms by which p53 may positively or negatively affect DNA repair in cells are not fully understood and likely involve complex interactions between many intracellular signals. Reports that p53 deficits act synergistically with MMR deficiency to determine cisplatin resistance in some cell lines (Branch et al. 2000), suggest that the unique combination of p53 deficiency and MMR deficiency in some cells may create conditions conducive to resistance. Accordingly, while p53 deficiency alone or MMR deficiency alone may not be able to account for cisplatin resistance, it cannot be completely ruled out that the combination of these two alterations may give rise to a resistant phenotype. Nonetheless, an examination of HCR in p53-deficient and MMR-deficient cells is needed before any

definitive statements can be made about the contribution of p53 deficits to cisplatin resistance in these particular cells.

Effects of hypoxia. Studies have demonstrated differential regulation of p53 by hypoxia under varying experimental conditions. Some studies have shown that p53 activity is suppressed under hypoxic conditions in some murine and human tumour cells (Ashcroft et al. 2000, Zhang et al. 2007), whereas other studies have demonstrated stabilization and transcriptional activity of p53 in other hypoxic normal and tumour cells (Ashcroft et al. 2000, Graeber et al. 1994, Koumenis et al. 2001). This differential effect of hypoxia on p53 activity appears to depend on the cell types examined, the presence of DNA damage and the severity and duration of the hypoxic stress employed in these studies. Some studies have examined p53 activity under conditions of severe hypoxia ($\leq$0.02% O_2), whereas other studies have looked at higher oxygen tensions. It has been suggested that the severity of oxygen deprivation (in addition to the length of stress), may change the profile of genes transactivated or transrepressed by p53 (Giaccia, personal communication). Furthermore, hypoxia signals both p53-dependent and p53-independent changes in the cell and it would appear that outcomes such as DNA repair or cell death under such conditions depend on a complex interaction of these hypoxia-induced changes. The current work showed no difference in HCR of the cisplatin-damaged reporter gene in $LFS^{p53-/-}$, $LFS^{p53+/-}$ or primary normal human fibroblasts (p53+/+) under hypoxic versus normoxic conditions suggesting that heterozygous and homozygous p53 mutations in these cells do not influence the effects of hypoxia on repair of cisplatin damaged DNA.

5.2 Host Cell Reactivation in SV40-transformed and Primary Human Normal Fibroblasts

Effects of SV40-transformation. We determined that the HCR values for the primary normal cell lines were not significantly different from each other (analysis not shown) and therefore pooled the results for all primary lines. It can be seen that under normoxic conditions HCR of the cisplatin-damaged reporter gene was significantly enhanced in SV40-transformed GM637F cells relative to primary normal fibroblasts at 40 h post-infection. However, scoring for β-gal activity at an earlier time point (24 h post-infection) did not reveal any significant differences in HCR between GM637F cells and primary normal fibroblasts (see Chapter 4), suggesting that the enhanced HCR of the cisplatin-damaged reporter gene in SV40-transformed GM637F cells at 40 h after infection results from additional repair occurring in these cells later than 24 h after infection. Our result of increased HCR in SV40-transformed cells suggests that SV40-transformed cells may be more resistant to cisplatin. However, other investigators have reported increased cisplatin sensitivity in SV40-transformed normal fibroblasts compared to primary normal fibroblasts (Furuta et al. 2002). This broad (and contradictory) range of responses between p53-compromised SV40-transformed cells and p53-proficient primary cells is similarly mirrored in the literature on the effect of p53 deficiency in tumour cell responses to cisplatin. At present, it appears that the effects of altered p53 activity on the response of cells to cisplatin may be cell type specific (as reviewed in Kartalou and Essigmann 2001). Nonetheless, these results provide insight into the possible consequences of transformation in normal cells, suggesting that enhanced repair of cisplatin-damaged DNA may be one outcome. Studies have already demonstrated that

transformation results in the cell's ability to bypass normal growth controls leading to an indefinite proliferative capacity and enhanced survival potential (Ahuja et al. 2005). In contrast, primary cells have a finite life span and can be passaged only a limited number of times before undergoing growth arrest and senescence. Given p53's role in cell cycle regulation, it is likely that compromised p53 contributes to the expanded proliferative capacity of SV40-transformed cells; however, less is known about the possible role of compromised p53 in enhancing repair of cisplatin-damaged DNA in SV40-transformed cells. Our results with the LFS cells suggest that p53 alteration may not be the critical alteration that confers a repair advantage in SV40-transformed cells. However, it is possible that p53 abrogation rather than p53 mutation and/or other cell changes induced by SV40-transformation (such as abrogated pRb), may be important in conferring a repair advantage and may collaborate with MMR deficits in tumour cells to produce cisplatin resistance.

Effects of hypoxia. HCR in SV40-transformed GM637F cells, but not primary cells was significantly reduced under hypoxic conditions (24 h pre-infection and 40 h post-infection) versus normoxic conditions. Similarly, reduced HCR in SV40-transformed murine 3340 cells has been reported under hypoxic and low pH conditions at 24 h after infection (Yuan et al. 2000). However, we have subsequently examined HCR in GM637F cells at 24 h post-infection under conditions of hypoxia and low pH and no longer observe a reduction in repair under these conditions (see Chapter 4). Taken together, these results indicate that hypoxia adversely affects HCR of cisplatin-damaged

DNA specifically in SV40-transformed cells and detection of this impairment in DNA repair requires hypoxia treatment longer than 24 h.

No significant effects of hypoxia (24 h pre-infection and 40 h post-infection) were detected in any of the primary cell lines. Similarly, no significant differences in HCR under normoxic versus hypoxic conditions were observed when results for all primary fibroblasts were pooled. Previous work within our lab (Dregoesc 2007, unpublished results), suggests that HCR of UVC-damaged DNA in GM9503 cells is significantly enhanced under conditions of hypoxia and acidosis (pH 6.5) versus normoxic conditions when cells are examined at 24 h. However, similar to the present findings, no differences in repair were detected when these cells were treated with hypoxia alone and scored at 40 h after infection. Therefore, we subsequently examined HCR at 24 h in GM9503 cells under hypoxic and acidic conditions (post-infection only, see Chapter 4), given the possibility that low pH conditions coupled with hypoxia and examining cells at an earlier time point may bring out differences in HCR. Under these conditions, we did not observe any effects of hypoxia and low pH on HCR of cisplatin-damaged DNA in GM9503 cells.

5.3 Host Cell Reactivation in MMR-deficient and MMR-proficient Human Adenocarcinoma Cells

Effects of MMR status. Under normoxic conditions, HCR of the cisplatin-damaged reporter gene was significantly enhanced in hMLH1-deficient HCT116 cells relative to hMLH1-proficient HCT116+ch3 cells consistent with a previous report (Cenni

et al. 1999). In another hMLH1-deficient cell line, Branch et al. (2000) found that abrogated p53 largely determined cisplatin resistance. By contrast, the hMLH1-deficient HCT116 cells used in the present work express wildtype p53 and loss of hMLH1 alone was sufficient to confer cisplatin resistance. However, the HCT116 cell line tested is an immortalized line and it cannot be ruled out that specific defects acquired through immortalization or spontaneous mutation (given the inherent genomic instability of MMR-deficient cells), may also make an important contribution to cisplatin resistance in this cell line. In addition, the hMLH1 protein is still present in HCT116 cells although it is truncated and may have important effects in the cell. For example, the mutated protein may still bind to damaged DNA sites, but may not be doing the same thing as the non-mutated protein or the mutation may produce a dominant effect in the cell. Furthermore, wildtype hMLH1 activity in HCT116+ch3 cells is restored via chromosome transfer and little is known about the effects of additional genes in these cells. Therefore the relative cisplatin sensitivity of HCT116+ch3 cells may be due to one of many genes on the transferred chromosome.

Cenni et al. (1999) have reported that HCR of a cisplatin-damaged plasmid-borne reporter gene was significantly enhanced in hMSH2-deficient HEC59 human endometrial carcinoma cells relative to hMSH2-proficient HEC59+ch2 cells. In contrast, we observed that HCR of our cisplatin-damaged reporter gene was reduced in hMSH2-deficient LoVo cells compared to hMSH2-proficient SW480 cells under normoxic and hypoxic conditions. This result corroborates our previous finding that loss of MSH2 alone could not account for resistance in isogenic murine fibroblasts (Chapter 2) and

further suggests that additional cell alterations may be required for cisplatin resistance in MSH2-deficient cells. Therefore, while fundamental cellular or genetic differences between SW480 and LoVo cells (which are derived from different parental lines), may account for their differing responses to cisplatin, the reverse relationship observed between MMR status and HCR suggests that LoVo cells lack the requisite genetic alterations that confer cisplatin resistance through collaboration with MMR deficiency.

Examination of both intrinsic and acquired resistance to cisplatin in several human ovarian carcinoma cell lines has shown that loss of MMR is not a primary contributing factor to resistance (Helleman et al. 2006, Massey et al. 2003). These studies suggest that other mechanisms (such as coexisting mutations), more readily account for cisplatin resistance in the examined cell lines. This raises the possibility that MMR deficiencies may be incidental to other more important alterations in some resistant cells. Our findings with hMSH2-deficient LoVo and SW480 control cells similarly suggest that loss of hMSH2 may not be a major determinant of cisplatin resistance relative to other genetic defects and cell mechanisms.

Effects of hypoxia. HCR of the cisplatin-damaged reporter gene was significantly reduced in hMLH1-deficient HCT116 cells under hypoxic conditions. Similarly, decreased repair of a UV-damaged reporter gene has been reported in an hMLH1-deficient RCneo human colon carcinoma cell line under hypoxic conditions (Yuan et al. 2000). HCR was also significantly reduced in hMLH1-proficient HCT116+ch3 cells. Mihaylova et al. (2003) has reported that hMLH1 is downregulated in hypoxia and we and others (Cenni et al. 1999) have shown that loss of hMLH1 is associated with

increased HCR of cisplatin-damaged DNA. Accordingly, a downregulation of hMLH1 under hypoxic conditions in HCT116+ch3 cells would be expected to enhance HCR. However, we have observed an opposite effect of reduced repair under hypoxic conditions in these cells. These contradictory findings may be reconciled by the fact that hMLH1 downregulation appears to be cell line specific and may depend in part on p53 deficiency. While downregulation of hMLH1 has been reported in HeLa cells under hypoxic conditions (Mihaylova et al. 2003), it has not been observed in other hypoxic cells (Koshiji et al. 2005). Notably, p53 is inactivated in HeLa cells but not in other cell lines that have been scored for hMLH1 expression in hypoxia (i.e. normal airway epithelial cells, MRC-5 fibroblasts) (Koshiji et al. 2005). The HCT116+ch3 cells used in the present work are p53 proficient; therefore, it is possible that hMLH1 may not be downregulated in these cells under hypoxic conditions given their p53 status and this may account for the failure to see enhanced HCR in these cells under hypoxic conditions.

Similarly, HCR was significantly reduced in hypoxic SW480 cells that are both MMR-proficient and p53-proficient. The MMR proteins hMSH2 and hMHS6 are known to be downregulated in hypoxia in a p53-dependent manner (Koshiji et al. 2005); however, this reduction in MMR activity did not correspond to an increase in HCR under hypoxic conditions. This result adds further support to our own findings and those of others that loss of MSH2 or MSH6 alone cannot account for cisplatin resistance (Claij and te Riele 2004, Chapter 2).

In contrast, no significant differences in repair under hypoxic versus normoxic conditions were observed in either hPMS2-deficient HEC1A cells or hMLH1- and

hPMS2-deficient SW48 cells. However, this does not preclude the possibility that differences in repair may be detectable if cells are treated with a combination of hypoxia and low pH conditions and scored earlier than 40 h post-infection, as shown for other cell types (Yuan et al. 2000).

Taken together, these results indicate that hypoxic effects vary with cell type. Interestingly, either a reduction in HCR or no difference in HCR under hypoxic versus normoxic conditions was observed in the panel of human tumour cells examined. These results suggest that hypoxia does not enhance repair in tumour cells. By contrast, we have previously shown both reduced and enhanced HCR in murine fibroblasts under hypoxic conditions emphasizing that important differences may exist between human and murine models (Chapter 2).

HCR of the AdCA35lacZ virus. HCR results obtained from HCT116, HCT116+ch3, LoVo and SW480 cells using an AdCA35*lacZ* viral construct with a murine (MCMV IE) promoter differed from the results obtained with the AdCA17*lacZ* virus employing a human (HCMV IE) promoter (discussed above). The only consistent finding was a significant reduction of HCR in hMSH2-deficient LoVo cells compared to hMSH2-proficient SW480 cells under hypoxic conditions; however, no other effects of MMR status were observed. In addition, no significant effects of hypoxia were observed in any of the cell lines tested, unlike the results we obtained using the HCMV IE promoter. The AdCA35 MCMV IE promoter has been shown to drive greater expression of β-gal in some human cells compared to the AdCA17 HCMV IE promoter (Addison et al. 1997). We have similarly observed both faster and greater β-gal expression using

AdCA35 in the cell lines examined. This faster and greater transcription of the *lacZ* gene in AdCA35 compared to AdCA17 may indicate that HCR of cisplatin damage in AdCA35 depends more heavily on the TCR pathway of NER (rather than GGR-NER), compared to HCR of cisplatin damage in AdCA17. Such promoter differences appear to affect overall differences in HCR both between cell lines and within cell lines under differing incubation conditions as indicated by our results with AdCA35 versus AdCA17. Therefore, use of a MCMV promoter may produce results that are not necessarily reflective of the normal situation in human cells where gene expression is controlled by a human promoter. However, it is possible that we may be able to detect similar differences in HCR with AdCA35 as with AdCA17 if β-gal is scored at earlier times after infection.

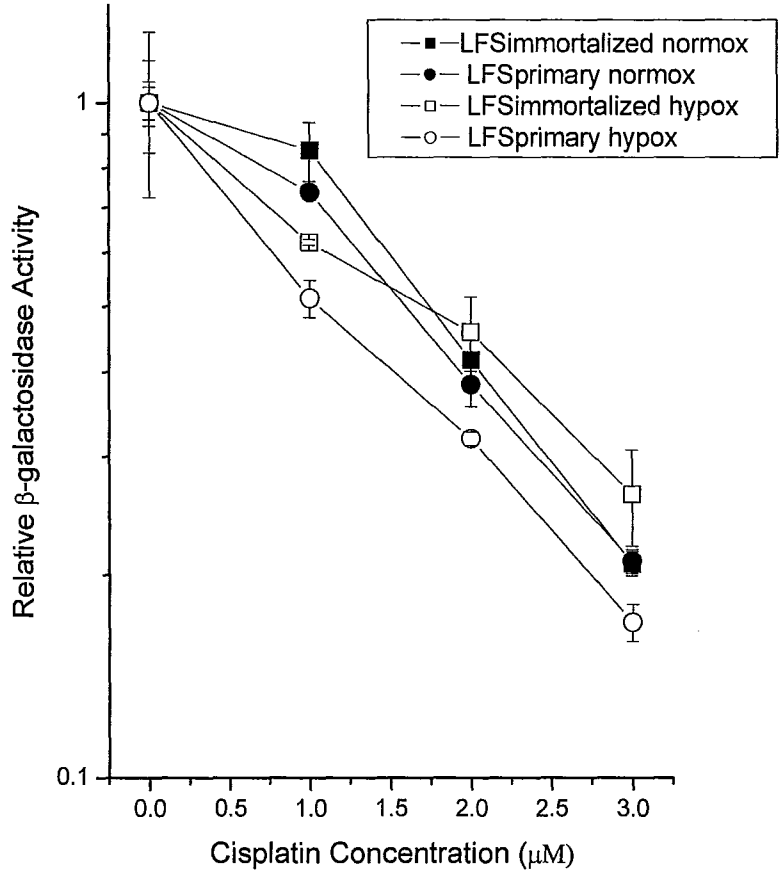

Figure 1. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in immortalized $LFS^{p53-/-}$ cells (■,□) and primary $LFS^{p53+/-}$ cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 135-190 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

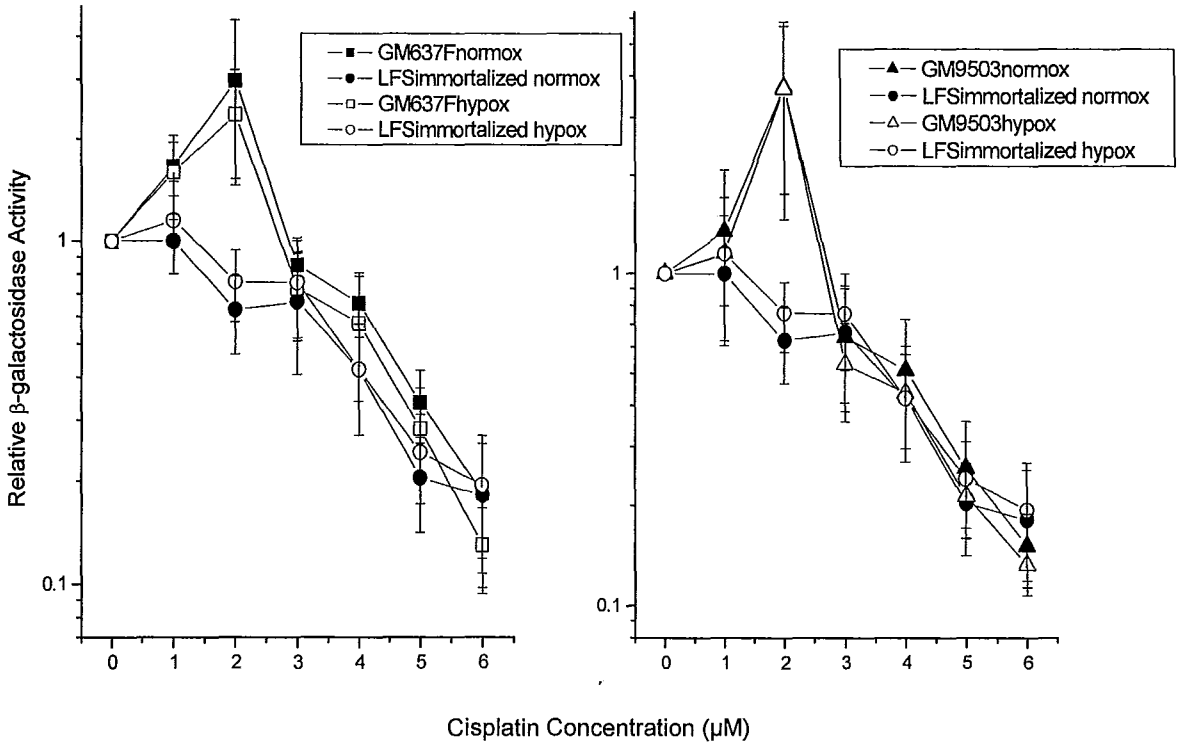

Figure 2. Left panel: HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in SV40-transformed normal GM637F cells (■,□) and immortalized LFS$^{p53-/-}$ cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Right panel: HCR in primary normal GM9503 cells (▲,△) and immortalized LFS$^{p53-/-}$ cells (●,○) under normoxic or hypoxic incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI 40-140 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 3 independent experiments each performed in triplicate.

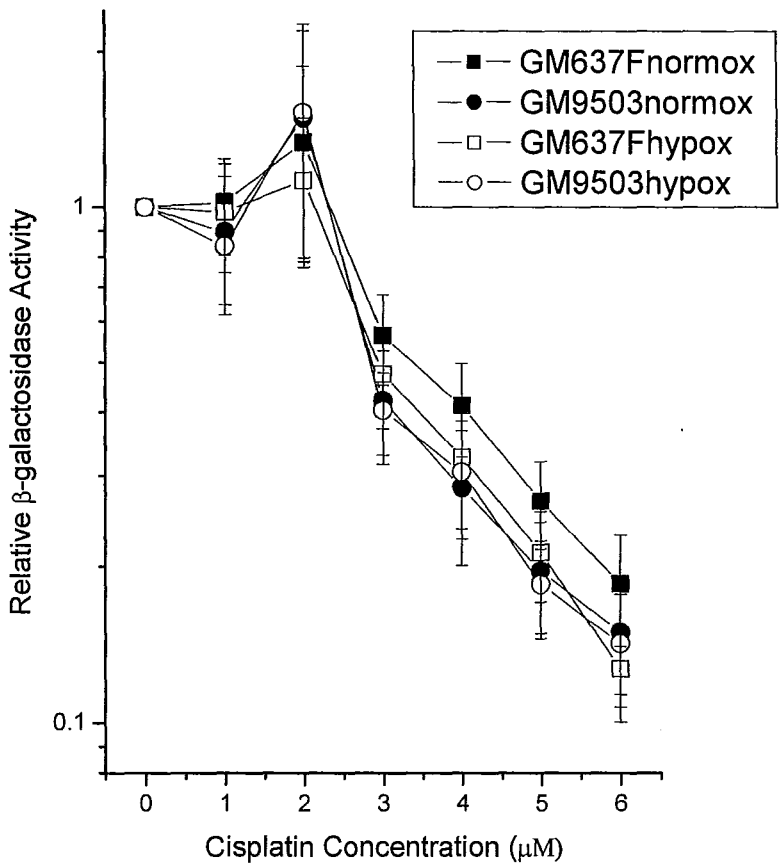

Figure 3. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in SV40-transformed normal GM637F cells (■,□) and primary normal GM9503 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40-140 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 10 independent experiments each performed in triplicate.

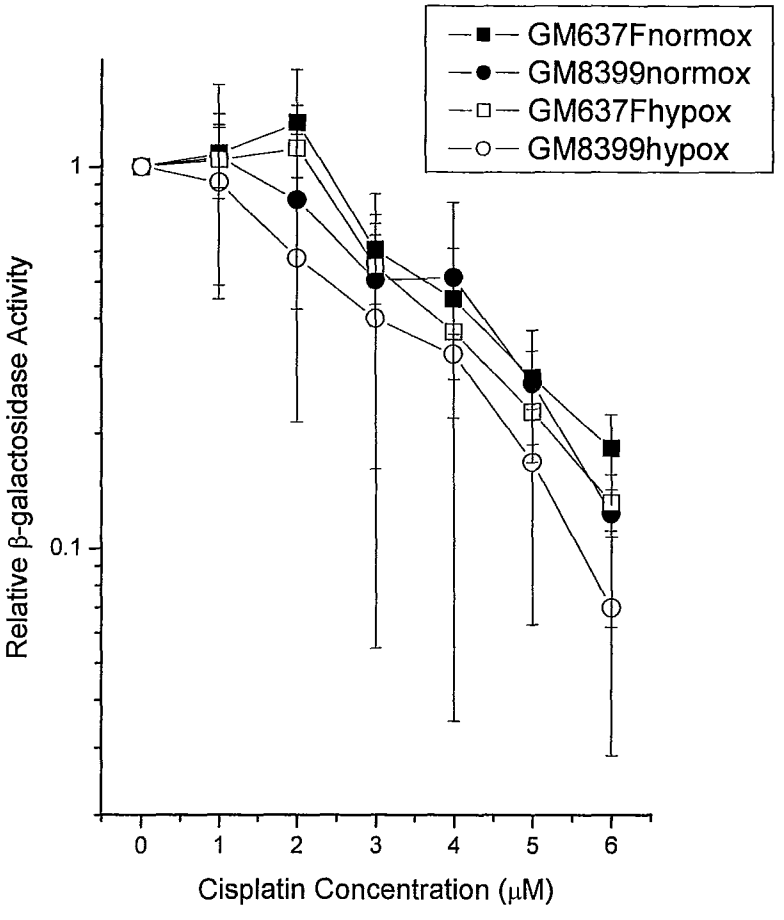

Figure 4. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in SV40-transformed normal GM637F cells (■,□) and primary normal GM8399 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40-140 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 3 independent experiments each performed in triplicate.

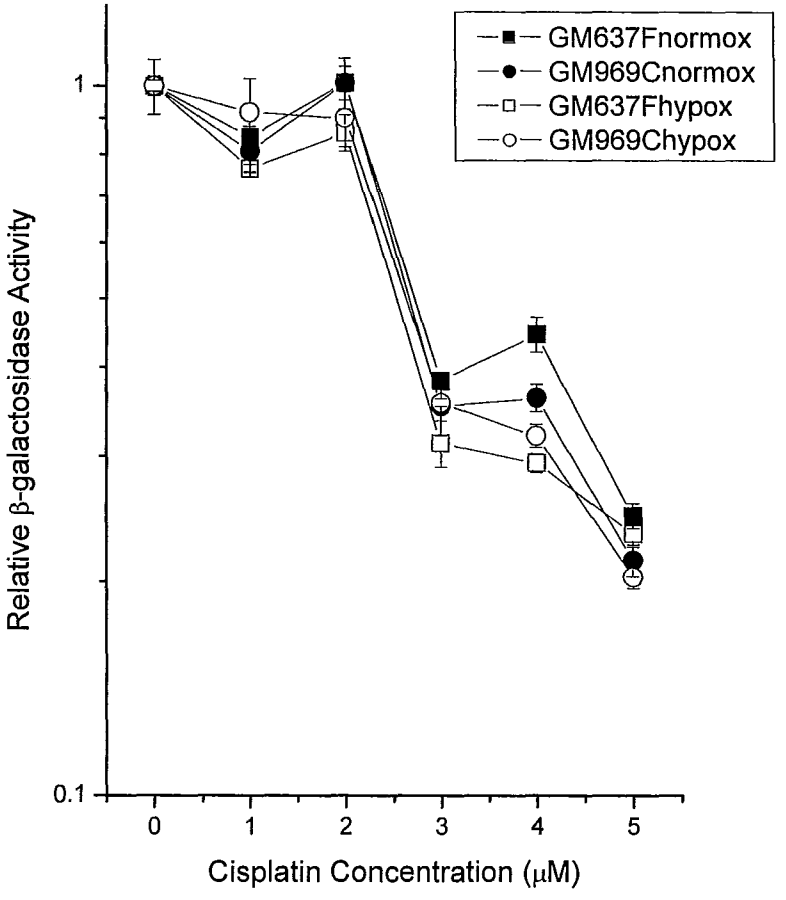

Figure 5. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in SV40-transformed normal GM637F cells (■,□) and primary normal GM969C cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40-140 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

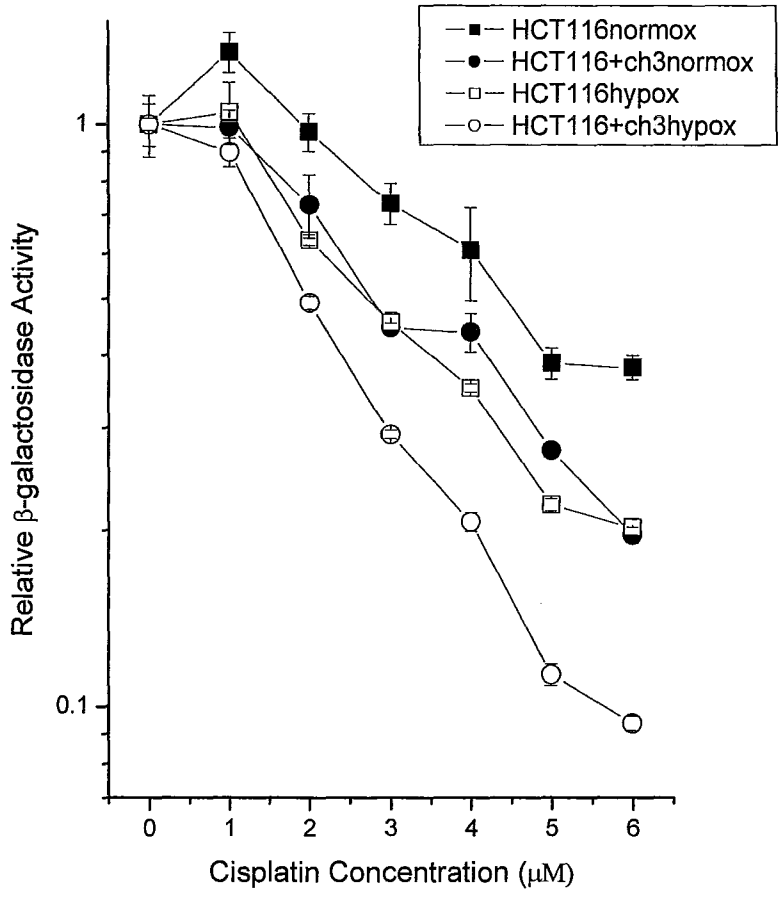

Figure 6. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in hMLH1-deficient HCT116 cells (■,□) and hMLH1-proficient HCT116+ch3 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 or 160 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

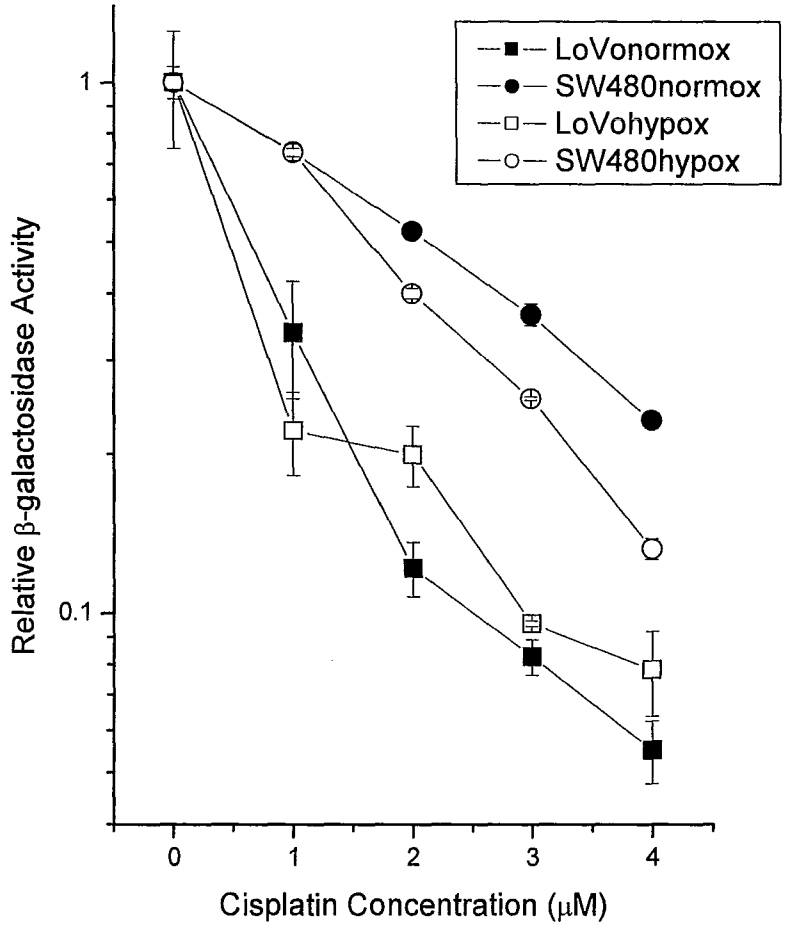

Figure 7. HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in hMSH2-deficient LoVo cells (■,□) and hMSH2-proficient SW480 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 or 160 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent one standard error.

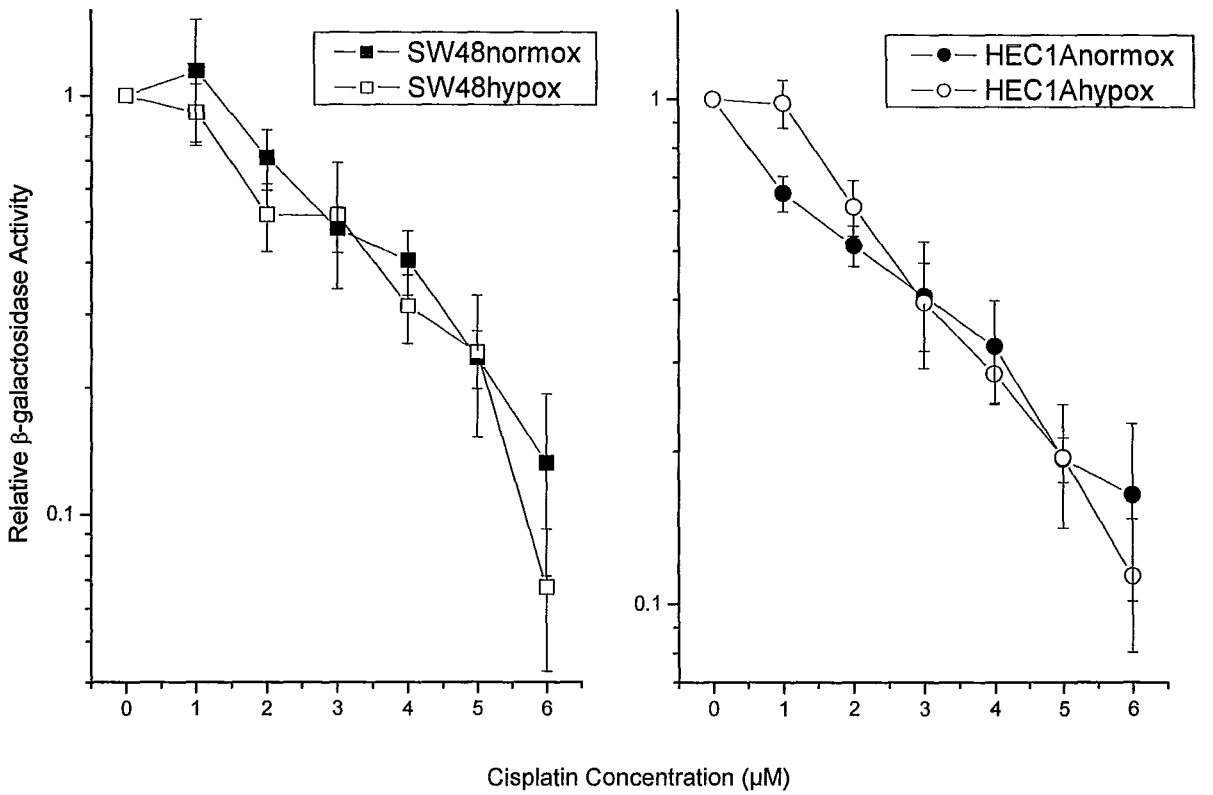

Figure 8. Left panel: HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in hMLH1- and hPMS2-deficient SW48 cells (■,□) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Right panel: HCR in hPMS2-deficient HEC1A cells (●,○) under normoxic or hypoxic incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 or 160 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 4 independent experiments each performed in triplicate.

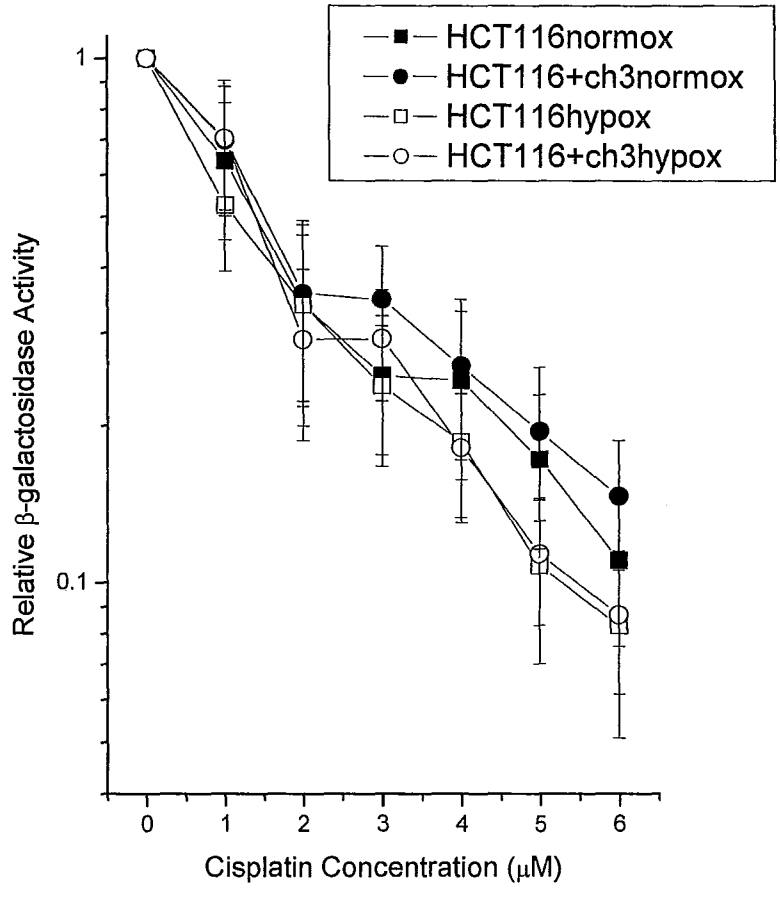

Figure 9. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in hMLH1-deficient HCT116 cells (■,□) and hMLH1-proficient HCT116+ch3 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Each point represents the average ± SE of 5 independent experiments each performed in triplicate.

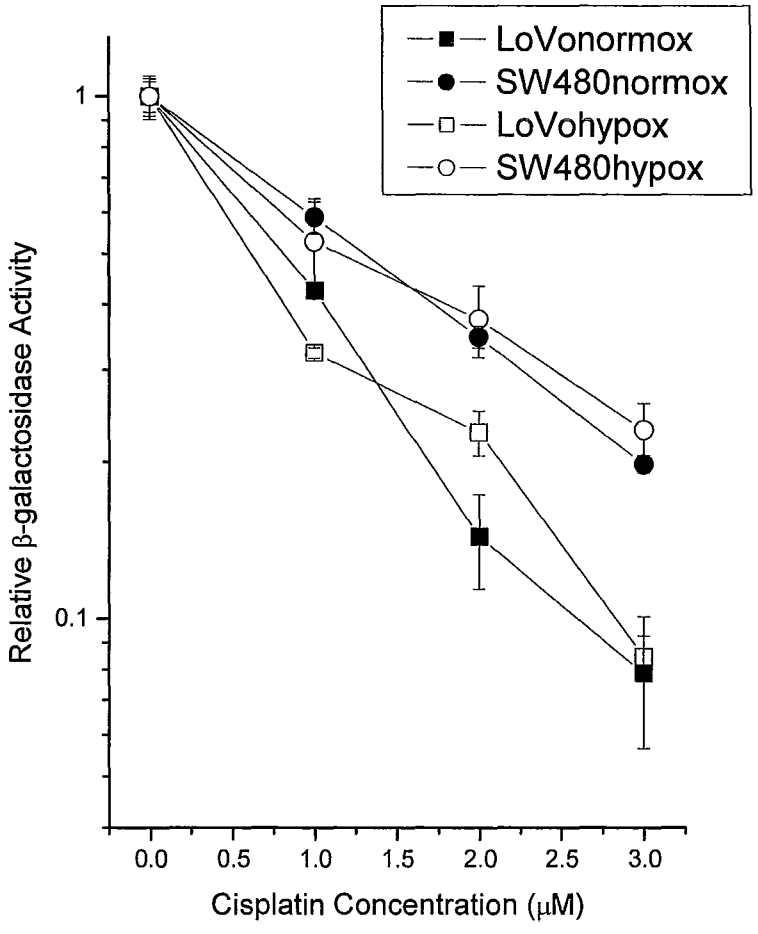

Figure 10. HCR of β-gal activity for the cisplatin-damaged AdCA35*lacZ* virus in hMSH2-deficient LoVo cells (■,□) and hMSH2-proficient SW480 cells (●,○) under normoxic (closed symbols) or hypoxic (open symbols) incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 40 pfu/cell. Cells were then incubated under normoxic or hypoxic conditions for 40 h and scored for β-gal activity. Results are shown for a single representative experiment, performed in triplicate. Bars represent standard error.

Table I. Human normal and adenocarcinoma cells used

Cell Line	Type	MMR/p53 status	Primary/ Transformed	Other Information
LFS087	Human LFS[a] cell	p53 +/-	Primary	Wildtype & mutant p53 expressed
LFS087	Human LFS cell	p53 -/-	Immortalized	Mutant p53 expressed
GM637F	Human NDF[b]	Abrogated p53	SV40-transformed	
GM9503	Human NDF	Functional p53	Primary	
GM969C	Human NDF	Functional p53	Primary	
GM8399	Human NDF	Functional p53	Primary	
HCT116	Human colon carcinoma cell	hMLH1- Functional p53	Immortalized	Truncated hMLH1
HCT116+ch3	Human colon carcinoma cell	hMLH1+ Functional p53	Immortalized	Derived from HCT116; Truncated hMLH1 + extra copy ch3 with wildtype hMLH1
LoVo	Human colon carcinoma cell	hMSH2- Functional p53	Immortalized	Non-isogenic pair
HEC1A	Human endometrial carcinoma cell	hPMS2-	Immortalized	
SW48	Human colon carcinoma cell	hMLH1- hPMS2-	Immortalized	
SW480	Human colon carcinoma cell	hMLH1+ hPMS2+ hMSH2+ Functional p53	Immortalized	Non-isogenic pair

[a]Li-Fraumeni Syndrome
[b]Normal Diploid Fibroblast

Table II. Average D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in immortalized ($LFS^{p53-/-}$) and primary ($LFS^{p53+/-}$) Li-Fraumeni Syndrome cells

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
$LFS087^{p53-/-}$	4	3.17±0.76	3.31±0.76
$LFS087^{p53+/-}$	4	2.52±0.71	2.43±0.72

Table III. p53 Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in immortalized ($LFS^{p53-/-}$) versus primary ($LFS^{p53+/-}$) Li-Fraumeni Syndrome cells

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
$LFS087^{p53-/-}$/ $LFS087^{p53+/-}$ (Normoxia)	4	1.33±0.28	0.32
$LFS087^{p53-/-}$/ $LFS087^{p53+/-}$ (Hypoxia)	4	1.47±0.30	0.22

Table IV. p53 Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in immortalized ($LFS^{p53-/-}$) Li-Fraumeni Syndrome cells versus human normal fibroblasts

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
$LFS087^{p53-/-}$/ GM637F (Normoxia)	3	0.732±0.118	0.15
$LFS087^{p53-/-}$/ GM9503 (Normoxia)	3	0.890±0.031	0.071
$LFS087^{p53-/-}$/ GM637F (Hypoxia)	3	0.929±0.081	0.47
$LFS087^{p53-/-}$/ GM9503 (Hypoxia)	3	1.02±0.06	0.75

Table V. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in immortalized ($LFS^{p53-/-}$) and primary ($LFS^{p53+/-}$) Li-Fraumeni syndrome cells under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
$LFS087^{p53-/-}$ Hypoxia/Normoxia	4	1.06±0.12	0.66
$LFS087^{p53+/-}$ Hypoxia/Normoxia	4	0.959±0.054	0.50

Table VI. Average D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 40 h in SV40-transformed and primary human normal fibroblasts

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia ± SE (μM)
GM637F (SV40)	11	4.14±0.63	3.37±0.48
GM9503 (primary)	11	3.49±0.58	3.68±0.49
GM8399 (primary)	3	2.83±1.44	2.47±1.36
GM969C (primary)	3	2.91±1.49	2.89±1.47
Pooled primary lines	17	3.27±0.49	3.32±0.45

Table VII. SV40-transformation Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 40 h in SV40-transformed versus primary human normal fibroblasts

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F/GM9503 (Normoxia)	11	1.27±0.10	**0.025**[a]
GM637F/GM8399 (Normoxia)	3	1.48±0.30	0.25
GM637F/GM969C (Normoxia)	3	1.44±0.29	0.26
GM637F/Primaries pooled (Normoxia)	17	1.34±0.09	**0.0021**[a]
GM637F/GM9503 (Hypoxia)	11	1.00±0.11	0.99
GM637F/GM8399 (Hypoxia)	3	1.47±0.31	0.27
GM637F/GM969C (Hypoxia)	3	1.34±0.39	0.48
GM637F/Primaries pooled (Hypoxia)	17	1.14±0.12	0.23

[a]significantly >1 by one-sample *t*-test (P<0.05)

Table VIII. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 40 h in SV40-transformed and primary human normal fibroblasts under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F Hypoxia/Normoxia	11	0.849±0.043	**0.0055**[a]
GM9503 Hypoxia/Normoxia	11	1.14±0.10	0.20
GM8399 Hypoxia/Normoxia	3	0.907±0.097	0.44
GM969C Hypoxia/Normoxia	3	0.991±0.006	0.30
Pooled primaries Hypoxia/Normoxia	17	1.07±0.07	0.25

[a]significantly <1 by one-sample *t*-test (P<0.05)

Table IX. Average D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in MMR-deficient and MMR-proficient human adenocarcinoma cells

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	No.	D_{37} Hypoxia ± SE (μM)
HCT116 (hMLH1-)	12	4.49±0.38	12	3.59±0.55
HCT116+ch3 (hMLH1+)	9	3.96±0.45	9	3.25±0.36
LoVo (hMSH2-)	8	1.86±0.47	9	2.34±0.52
SW480 (hMSH2+)	12	3.47±0.25	12	2.96±0.38
HEC1A (hPMS2-)	4	3.21±0.55	4	3.13±0.41
SW48 (hMLH1- & hPMS2-)	4	3.92±0.49	4	3.12±0.59

Table X. MMR Status Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in MMR-deficient versus MMR-proficient human adenocarcinoma cells

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
HCT116/HCT116+ch3 (Normoxia)	8	1.21±0.06	**0.010**[a]
LoVo/SW480 (Normoxia)	8	0.563±0.101	**0.0035**[b]
HCT116/HCT116+ch3 (Hypoxia)	8	1.18±0.11	0.15
LoVo/SW480 (Hypoxia)	8	0.721±0.083	**0.012**[b]

[a]significantly >1 by one-sample *t*-test (P<0.05)
[b]significantly <1 by one-sample *t*-test (P<0.05)

Table XI. Hypoxia Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* in MMR-deficient and MMR-proficient human adenocarcinoma cells under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
HCT116 Hypoxia/Normoxia	12	0.782±0.054	**0.0020**[a]
HCT116+ch3 Hypoxia/Normoxia	9	0.826±0.030	**0.00041**[a]
LoVo Hypoxia/Normoxia	8	1.30±0.31	0.36
SW480 Hypoxia/Normoxia	11	0.783±0.041	**0.00033**[a]
HEC1A Hypoxia/Normoxia	4	1.01±0.094	0.92
SW48 Hypoxia/Normoxia	4	0.784±0.099	0.12

[a]significantly <1 by one-sample *t*-test (P<0.05)

Table XII. Average D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* in MMR-deficient and MMR-proficient human adenocarcinoma cells

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	No.	D_{37} Hypoxia ± SE (μM)
HCT116 (hMLH1-)	5	2.52±0.66	5	2.20±0.38
HCT116+ch3 (hMLH1+)	5	3.00±0.70	5	2.09±0.40
LoVo (hMSH2-)	4	1.31±0.23	3	1.66±0.55
SW480 (hMSH2+)	5	2.34±0.81	4	2.43±0.50

Table XIII. MMR Status Effects with a Murine CMV IE Promoter: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* in MMR-deficient versus MMR-proficient human adenocarcinoma cells

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
HCT116/HCT116+ch3 (Normoxia)	5	0.847±0.061	0.067
LoVo/SW480 (Normoxia)	4	0.652±0.134	0.081
HCT116/HCT116+ch3 (Hypoxia)	5	1.12±0.19	0.57
LoVo/SW480 (Hypoxia)	3	0.567±0.084	**0.036**[a]

[a]significantly <1 by one-sample *t*-test (P<0.05)

Table XIV. Hypoxia Effects with a Murine CMV IE Promoter: Relative D_{37} values from HCR of cisplatin-damaged AdCA35*lacZ* in MMR-deficient and MMR-proficient human adenocarcinoma cells under hypoxic versus normoxic incubation conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
HCT116 Hypoxia/Normoxia	5	0.965±0.124	0.79
HCT116+ch3 Hypoxia/Normoxia	5	0.753±0.092	0.055
LoVo Hypoxia/Normoxia	3	1.15±0.19	0.50
SW480 Hypoxia/Normoxia	4	1.04±0.19	0.83

CHAPTER 4

A Comparison of Repair of UV-induced and Cisplatin-induced DNA Damage in SV40-transformed and Primary Normal Human Fibroblasts Under Normoxic and Hypoxic Plus Low pH Conditions

1.0 Abstract

Cisplatin and UVC irradiation both cause damage to DNA that is primarily repaired by the nucleotide excision repair (NER) pathway. In addition, repair of cisplatin interstrand crosslinks requires the homologous recombination repair (HRR) pathway. Rapidly growing tumours frequently outstrip their blood supply resulting in a hypoxic tumour environment. Hypoxia has been shown to influence DNA repair in cells and it has been suggested that low pH conditions may also be necessary to bring out differences in repair (Yuan et al. 2000). Hypoxia-induced repression of key repair factors including the HRR proteins RAD51 and BRCA1 has also been demonstrated (Bindra et al. 2005a, Bindra et al. 2005b). It is unclear whether the involvement of HRR in the repair of cisplatin damage affects the extent of repair of cisplatin- versus UVC-damaged DNA under normoxic and/or hypoxic conditions. We have examined host cell reactivation (HCR) of a cisplatin-damaged or UVC-damaged AdCA17*lacZ* viral construct in human primary and SV40-transformed normal fibroblasts under normoxic or hypoxia plus low pH conditions. We show that HCR of the UVC-damaged reporter gene is reduced in SV40-transformed GM637F cells compared to primary normal human fibroblasts under conditions of normoxia. In contrast, we show no reduction in HCR of the cisplatin-damaged reporter gene in GM637F cells compared to primary human fibroblasts indicating a differential effect of SV40-transformation on repair of cisplatin-damaged DNA compared to UVC-damaged DNA. This difference may reflect the contribution of HRR to repair of cisplatin-damaged DNA given the higher frequency of HRR previously reported in SV40-transformed cells versus primary cells (Finn et al. 1989). Previous

studies have reported that HCR of a UV-damaged plasmid-borne reporter gene is significantly reduced in 3340 transformed murine fibroblasts and in human RKO (RCneo) colorectal carcinoma cells under hypoxia plus low pH conditions suggesting that repair functions may be impaired in the hypoxic tumour microenvironment (Yuan et al. 2000). In contrast, the results of the present study show no reduction in HCR of a UVC-damaged or a cisplatin-damaged reporter gene in SV40-transformed and primary normal human fibroblasts under similar conditions of hypoxia and low pH. These results suggest that DNA repair of UVC-induced DNA damage is differentially affected by hypoxia and low pH depending on cell type.

2.0 Introduction

Studies of DNA repair have been invaluable in elucidating how cells respond to carcinogens in the environment such as solar UV-irradiation and have been equally useful in examining cell responses to conventional cancer treatments like the platinum-based drug cisplatin (Lee et al 2004, Pitsikas et al. 2005, Cenni et al. 1999, Sheibani et al. 1989). UV-irradiation and cisplatin cause different types of damage in DNA. Cisplatin produces both intrastrand and interstrand DNA crosslinks between adjacent or proximal purine bases (i.e. guanine and adenine). Intrastrand adducts constitute the majority of these crosslinks (≥85%), while interstrand adducts comprise a smaller proportion (1-3%) (Chaney et al. 2004). In contrast, UV-irradiation of DNA results in pyrimidine dimer products which occur between adjacent thymine and cytosine bases (Matsumura and Ananthaswamy 2004). These include cyclobutane pyrimidine dimers (CPDs) and 6-4

photoproducts. Despite these differing forms of DNA damage, both UV-induced and cisplatin-induced intrastrand lesions are repaired primarily by the NER pathway (reviewed in Costa et al. 2003, Furuta et al. 2002). Briefly, this pathway involves recognition of damaged bases, unwinding of the DNA helix surrounding the damaged site, excision of damaged nucleotides and DNA synthesis to fill in the gap. In addition to the NER pathway, repair of cisplatin-induced DNA damage, specifically interstrand adducts, requires the HRR pathway (reviewed in Lodish et al. 2004). Briefly, free 3' ends are created at the site of the double strand break and RAD51 assembles into nucleoprotein filaments along these ends. One filament base pairs with a complementary sequence in a homologous chromosome and the sequence information is used to elongate the free 3'end. The remaining free 3'end is elongated upon pairing with the newly polymerized strand. Repair of UV damage is less dependent on HRR since interstrand DNA crosslinks occur less frequently following UV-irradiation and double strand breaks are not typically associated with UV damage (Friedberg et al. 1995).

It is unclear whether the involvement of HRR in the repair of cisplatin interstrand adducts results in important differences in the repair of cisplatin-damaged versus UV-damaged DNA. It is also unknown to what extent HRR may contribute to HCR of β-gal expression for a cisplatin-damaged reporter gene. A higher frequency of HRR has been reported in SV40-transformed and immortalized cells relative to non-transformed cells (Finn et al. 1989, Thyagarajan et al. 1996). In addition, we have previously shown that HCR of a cisplatin-damaged reporter gene is enhanced in SV40-transformed cells relative to primary cells when cells are examined at 40 h post-infection (Chapter 3). By contrast,

previous work has found that repair of UVC-damaged DNA is reduced in SV40-transformed cells compared to primary cells (Bowman et al. 2000, Rainbow 1989); likewise, a reduced capacity of SV40-transformed cells to support viral DNA synthesis following UVC-irradiation has been reported previously (McKay et al. 1997). These findings suggest that the repair capacity of SV40-transformed cells compared to primary normal human fibroblasts depends on the type of DNA damage. Another source of repair differences may be the degree of DNA distortion produced by cisplatin and UVC irradiation, since each type of DNA damage may be differentially recognized or processed resulting in subsequent differences in the repair of cisplatin versus UVC lesions.

Additionally, cell responses to DNA damaging agents are influenced by the hypoxic tumour microenvironment that is characterized by regions of fluctuating or chronic hypoxia, low pH and nutrient deprivation. Reduced host cell reactivation of a UV-damaged reporter gene in transformed murine fibroblasts and human RKO (RCneo) colorectal carcinoma cells under hypoxia and low pH conditions has been reported (Yuan et al. 2000). This reduction in repair was also associated with increased mutagenesis. However, results from our lab suggest that HCR of UVC-damaged DNA may not be reduced in human normal cells under conditions of hypoxia and low pH; in fact, HCR may be enhanced in primary normal fibroblasts under these conditions (Dregoesc 2007, unpublished results). In comparison, little is known about the repair of cisplatin-damaged DNA under hypoxic conditions and it is uncertain whether the results from hypoxia studies with UV can be generalized to the repair of cisplatin-damaged DNA in hypoxic

cells. Hypoxic repression of RAD51 and BRCA1 (two repair proteins involved in HRR), has been reported previously and this reduction corresponds to a functional decrease in HRR (Bindra et al. 2005a, Bindra et al. 2005b). Since HRR is more integral to the repair of cisplatin adducts, downregulation of HRR may have a more detrimental impact on repair of cisplatin-damaged DNA relative to repair of UV-damaged DNA under hypoxic conditions. The importance of HRR to the repair of cisplatin-damaged DNA is evident in studies with Fanconi's Anemia (FA) cells that are believed to be deficient in interstrand crosslink repair (Fujiwara 1982, Fujiwara et al. 1977, Sasaki and Tonomura 1973). FA cells are more sensitive to cisplatin and tolerate fewer cisplatin interstrand crosslinks than control fibroblasts (Plooy et al. 1985). Similarly, deficient gene-specific repair of cisplatin interstrand crosslinks compared to normal controls has been reported in FA group A cells (Zhen et al.. 1993). Taken together, these findings suggest that persisting platinum interstrand crosslinks in the genome may contribute to cytotoxicity in these cells.

We have previously shown that HCR of cisplatin-damaged DNA is reduced in SV40-transformed GM637F cells under hypoxic versus normoxic conditions when cells are scored at 40 h post-infection (Chapter 3). However, we observed no effects of hypoxia on HCR of the cisplatin-damaged reporter gene in primary GM9503 cells or GM8399 cells at 40 h.

We have used a host cell reactivation (HCR) assay to examine repair of both a cisplatin-damaged and UVC-damaged *lacZ* reporter gene encoding β-galactosidase at 24 h in SV40-transformed and primary human normal fibroblasts under normoxic

conditions and hypoxia plus low pH conditions. This assay allows for a possible examination of HRR in addition to an evaluation of NER since multiple copies of the viral construct containing the reporter gene are infected into cells. Therefore, recombination can occur between different copies of the *lacZ* reporter gene in regions where DNA damage is non-overlapping. We sought to address whether the operation of HRR in the repair of cisplatin damage might contribute to differences in the repair of cisplatin-damaged versus UVC-damaged DNA. We also sought to examine whether hypoxia and low pH conditions could bring out repair differences in some cell lines relative to normoxic conditions.

3.0 Materials and Methods

3.1 Cell Lines

The SV40-transformed (GM637F) and primary (GM9503 and GM8399) human normal cell lines used in the present work were purchased from the National Institute of General Medical Sciences, Camden, NJ and are described in Table I. Cell cultures were grown in a humidified incubator at 37°C in 5% CO_2 and were cultured in monolayer. All cells were maintained in alpha-minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% antibiotic-antimycotic (Gibco BRL, USA).

3.2 Cisplatin Treatment to Virus

The recombinant adenovirus AdCA17 (AdHCMV*lacZ*) used in this work was obtained from Dr. F. L. Graham at McMaster University, Hamilton ON. The viral construct consists of an *E. coli lacZ* reporter gene encoding β-galactosidase (β-gal) under the control of a human cytomegalovirus early promoter (HCMV-IE) and a SV40 polyadenylation signal (Addison et al. 1997). All three components have been inserted into the deleted early region 1 (E1) of the viral genome. Deletion of the viral E1 gene renders the virus incapable of replicating in mammalian cells except where the E1 gene is expressed in trans.

Stock cisplatin (3333 μM) used in this work was purchased as a 1 mg/mL solution (Faulding, Montreal, QC). Stock cisplatin was serially diluted in a solution of α-MEM and low chloride PBS (50 mM Cl^-) to activate the drug. A viral suspension was then prepared in 1.8 mL of the low chloride solution in a 35 mm diameter Petri dish (Falcon, USA) on ice. 20 μL aliquots of each cisplatin concentration were added to separate microtubes followed by the addition of 200 μL of viral suspension to each tube, producing final cisplatin concentrations of 1-6 μM. The treated virus was incubated for 12 h at 37°C after which time cisplatin damage to the viral DNA was stopped with the addition of 1 mL of unsupplemented α-MEM to each tube.

3.3 UVC-irradiation of Virus

A viral suspension was prepared in 1.8 mL of PBS in a 35 mm diameter Petri dish on ice. While being stirred, the viral suspension was irradiated with UVC light (primarily

at 254 nm) from a germicidal lamp (General Electric, model G8T5) at a fluence of 2 J/m^2 as measured by a Black-Ray ultraviolet meter (model J225, Ultraviolet Products, Upland, CA). The viral suspension was exposed to the UVC lamp for increasing lengths of time resulting in UVC fluences ranging from 0 J/m^2 to 600 J/m^2. After each exposure, 200 µL of viral suspension was removed and added to 1 mL of unsupplemented α-MEM in separate microtubes kept on ice.

3.4 Low pH Media

Low pH media was prepared by adding 25 mM of HEPES free acid [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] (Gibco Laboratories, Grand Island, NY) and 25 mM of MOPS free acid [3-(N-Morphollno)propanesulfonic acid] (BioShop Canada Inc., Burlington, ON) to 50 mL of supplemented α-MEM. The pH of the solution was measured with a pH meter (Corning model 440, NY) and was adjusted to 6.5 with the drop wise addition of prepared 1N HCL or 1N NaOH.

3.5 Host Cell Reactivation Assay

Cells were seeded at densities ranging from 2.0x10^4 to 3.5x10^4 cells per well (depending on the cell line) in 96-well tissue culture plates (Falcon, Lincoln Park, NJ). Cells were then incubated for 24 h at 37°C under normoxic conditions. Following incubation, media was aspirated from the wells and cells were infected with 40 µL of cisplatin-treated, UVC-irradiated or non-treated virus at a MOI of 80 (transformed cells) or 140 (primary cells) plaque forming units (pfu) per cell. After 90 min of viral

adsorption, excess viral solution was aspirated and supplemented growth media was added to the normoxic treatment plates while supplemented low pH media was added to the hypoxic treatment plates. Cells were then incubated at 37°C under normoxic (21% O_2) or hypoxic (1% O_2 as described in Chapter 2) conditions for 24 h and then harvested with the addition of 1 mM CPRG (Roche, Indianapolis, IN) in 0.01% Triton X-100, 1 mM $MgCl_2$ and 100 mM phosphate buffer (pH 8.3). β-gal activity was scored using a 96-well plate reader (EL340 Bio Kinetics Reader, Bio-Tek Instruments) measuring absorbance at a wavelength of 570 nm. β-gal activity in cells was expressed relative to untreated controls (± standard error) as described in Chapter 2. Each HCR experiment for a given cell line consisted of triplicate determinations for each cisplatin treatment of the virus and a minimum of three independent HCR experiments were performed for each cell line.

4.0 Results

4.1 Effects of SV40-transformation on Host Cell Reactivation of Cisplatin- and UVC-damaged AdCA17 in Human Normal Fibroblasts

The relative β-gal activity of the cisplatin-damaged reporter gene at 24 h after infection with AdCA17 for SV40-transformed and primary human normal fibroblasts is shown in Figure 1. The relative β-gal activity of the UVC-damaged reporter gene at 24 h after infection with AdCA17 is shown in Figure 2. D_{37} is the cisplatin concentration that reduces the expression of β-gal activity to 37% and is used as a measure of HCR. No significant differences in HCR of the cisplatin-damaged reporter gene were observed

between SV40-transformed GM637F cells and primary GM9503 or GM8399 cells (see Tables II and III). Pooled results for both primary normal cell lines also showed no differences in HCR between transformed GM637F cells compared to the primary normal fibroblasts. In contrast, HCR of the UVC-damaged reporter gene was significantly reduced ($P < 0.05$) in SV40-transformed GM637F cells compared to primary human fibroblasts cells under both incubation conditions (see pooled results in Tables IV and V). The reduction in HCR of the UV-damaged reporter gene in SV40-transformed cells compared to primary normal cells was similar for GM9503 and GM8399 with relative HCR values of 0.56 and 0.53 respectively.

4.2 Effects of Hypoxia and Low pH on Host Cell Reactivation of Cisplatin- and UVC-damaged AdCA17 in Human Normal Fibroblasts

The relative D_{37} values for HCR of the cisplatin-damaged reporter gene in all cell lines under hypoxic plus low pH conditions (24 h post-infection) versus normoxic conditions are presented in Table VI. It can be seen that HCR of the cisplatin-damaged reporter gene was not significantly reduced under hypoxic plus low pH conditions versus normoxic conditions in the SV40-transformed GM637F cells or when results for the primary normal fibroblasts were pooled. When the primary GM8399 line is considered separately, a reduction in HCR under hypoxia and low pH conditions is observed however, the pooled primary cell results suggest that this may be an anomalous result.

The relative D_{37} values for HCR of the UVC-damaged reporter gene under hypoxic plus low pH conditions (24 h post-infection) compared to normoxic conditions

are presented in Table VII. No significant effects of hypoxia and low pH on HCR of the UVC-damaged reporter gene were observed in any of the cell lines or when results for all primary normal cells were pooled, similar to our findings for HCR of the cisplatin-treated reporter gene.

5.0 Discussion

5.1 Effects of SV40-transformation on Host Cell Reactivation of Cisplatin- and UVC-damaged AdCA17 in Human Normal Fibroblasts

In the present chapter we show that when β-gal activity is scored at 24 h after infection HCR of the UVC-damaged reporter gene is reduced in SV40-transformed GM637F cells compared to primary normal human fibroblasts under conditions of normoxia. A similar reduction of HCR in GM637F compared to primary normal human fibroblasts is detected when β-gal activity is scored at 40 h after infection (Zacal 2007, personal communication). These results are consistent with previous reports that show GM637 cells have deficient NER (GGR at least and possibly also TCR) of UVC-induced DNA lesions due to the SV40- abrogation of p53 and pRb (Rainbow 1989, Bowman et al. 2000). In contrast to the reduced HCR for the UVC-damaged reporter gene in GM637F cells we show no reduction in HCR of the cisplatin-damaged reporter gene in GM637F cells compared to primary human fibroblasts when scoring for β-gal at 24 h after infection. In addition, HCR of the cisplatin-damaged reporter gene was significantly enhanced in SV40-transformed GM637F cells relative to primary normal human fibroblasts when cells were scored for β-gal at 40 h after infection (Chapter 3).

Taken together, our comparisons of HCR in GM637F cells and primary normal fibroblasts suggest that SV40-transformed normal cells are less efficient at repairing UVC-damaged DNA but not cisplatin-damaged DNA, compared to primary normal cells. This difference may reflect the operation of HRR in the repair of cisplatin-damaged DNA. Specifically, a higher frequency of HRR has been reported in SV40-transformed human normal cells compared to primary cells (Finn et al. 1989). Likewise, higher levels of HRR have been reported in SV40-transformed primate cells and immortalized murine fibroblasts relative to their non-transformed and non-immortalized parental lines (Thyagarajan et al. 1996). Given the greater contribution of HRR to the repair of cisplatin damage, it is possible that elevated levels of HRR in SV40-transformed GM637F cells may have masked any deficits in the repair of cisplatin-damaged DNA by NER resulting in an overall level of repair comparable to that in primary cells. Furthermore, elevated HRR in GM637F cells may account for the enhanced repair of cisplatin damage relative to primary controls that we have observed at 40 h post-infection (Chapter 3).

A previous study examining HCR of a methylene blue plus visible light (MB+VL)-damaged reporter gene has shown that HCR in transformed GM637 cells is greater than that in primary normal fibroblasts when cells are examined at 40-48 h after infection (Kassam and Rainbow 2007). MB+VL induces oxidative base damage in DNA primarily in the form of 8-OxoG lesions (7,8-dihydo-8-oxoguanine). The repair of these lesions involves the base excision repair (BER) pathway. Taking these results together with our HCR results for cisplatin- and UVC-damaged DNA suggests that abrogation of

p53 and/or pRb by SV40-transformation may affect NER, HRR and BER in different ways.

SV40-transformation of cells inhibits the activities of p53 and pRb since large T antigen, a protein product of SV40 binds to both p53 and the pRb family of proteins preventing their activation of downstream effectors involved in cell cycle regulation and DNA repair. However, whether the binding of p53 to large T antigen fully suppresses all functions of p53 is controversial as some studies have found large amounts of unbound p53 in SV40-transformed cells and other studies have demonstrated some p53 activity in SV40-transformed cells (Hess and Brandner 1997, O'Neill et al. 1997). Both HRR (involved in repair of cisplatin adducts) and BER are thought to be regulated in part by p53. Several studies have suggested that wildtype p53 may suppress spontaneous HRR in some cells and correspondingly that abrogation or mutation of p53 results in elevated HRR (Linke et al. 2003, Mekeel et al. 1997, Saintigny et al. 1999). Other studies have reported that expression of the HRR protein RAD51 is repressed by wildtype p53 (Arias-Lopez et al. 2006). This downregulation of HRR function by p53 may explain previous observations of elevated HRR in SV40-transformed cells (Finn et al. 1989, Thyagarajan et al. 1996), in addition to our own observation of elevated HCR of cisplatin-damaged DNA in SV40-transformed cells compared to primary normal fibroblasts at 40 h post-infection. Although HRR functions to repair double strand breaks that can't be handled by other repair systems, inappropriate recombination activity may result in chromosomal abnormalities (i.e. translocations) leading to mutagenesis. Therefore, p53 may have evolved a role in downregulating HRR to circumvent mutagenesis.

In contrast, wildtype p53 appears to stimulate BER activity in some cells (possibly through direct interaction with components of the BER complex) and correspondingly, BER activity correlates closely with cellular p53 levels *in vitro* (Offer et al. 1999, Zhou et al. 2001). Reduced repair of methyl methanesulfonate-induced damage by BER in p53-deficient compared to p53-proficient cells has also been reported (Seo et al. 2002). However, work by others has suggested that p53-dependent activation of BER may depend on the DNA damaging agent. Of interest, Zurer et al. (2004) have reported that p53 enhances activation of a key BER glycosylase following gamma-irradiation whereas p53 was shown to downregulate glycosylase activity in response to nitric oxide. Additionally, no enhancement of glycosylase activity by p53 was observed following hydrogen peroxide treatment. The study by Kassam and Rainbow (2007) certainly indicate that BER of base damage induced by MB+VL is reduced in cells expressing wildtype p53 compared to cells in which p53 is abrogated. However, the mechanism responsible for this difference is unclear. It may be that p53 downregulates BER activity specifically in response to MB+VL treatment as seen for nitric oxide. Contrastingly, bound p53 may still be able to stimulate BER in SV40-transformed cells perhaps with the protein itself being involved (possibly through direct interaction with BER proteins), rather than acting as a transcriptional activator of other DNA repair genes.

A role for p53 in NER has been demonstrated in several studies. Reduced repair of UV-induced DNA damage has been reported in p53-defective cells relative to cells with wildtype p53 and this reduction in repair has been shown to correlate with reduced cell survival (Rainbow 1989, Smith et al. 1995, Li et al. 1996). Furthermore,

defects in p53 have been linked specifically to reductions in the GGR pathway of NER (Ford and Hanawalt 1995, Bowman et al. 2000) and GGR requires p53 to transcriptionally upregulate the XPC and p48XPE genes by binding to the p53 consensus sequence in the promoter regions of these genes (Adimoolam and Ford 2002, Hwang et al. 1999). The involvement of p53 in TCR-NER is more controversial although several studies have suggested a possible role for p53 in this pathway (Dregoesc et al. 2006, Mathonnet et al. 2003, Mathonnet et al. 2004, McKay et al. 2001, Therrien et al. 1999). Given that UVC damage is primarily repaired by NER, our findings that repair of UVC-induced DNA damage is reduced in SV40-transformed cells with abrogated p53 compared to primary cells expressing wildtype p53 is consistent with the demonstrated role of p53 in positively regulating NER.

5.2 Effects of Hypoxia and Low pH on Host Cell Reactivation of Cisplatin- and UVC-damaged AdCA17 in Human Normal Fibroblasts

Previous studies have reported that HCR of a UV-damaged plasmid-borne reporter gene is significantly reduced in 3340 transformed murine fibroblasts and human RKO (RCneo) colorectal carcinoma cells under hypoxia plus low pH conditions suggesting that repair functions may be impaired under conditions of the tumour microenvironment (Yuan et al. 2000). A similar reduction in HCR of a UVC-damaged recombinant adenovirus-borne reporter gene has also been detected in these cells (Dregoesc 2007, unpublished results). In contrast, the results of the present study show no reduction in HCR of a UVC-damaged or a cisplatin-damaged reporter gene in SV40-

transformed and primary normal human fibroblasts under similar conditions of hypoxia and low pH. These results suggest that DNA repair of UVC-induced DNA damage is differentially affected by hypoxia and low pH depending on cell type. Using a similar protocol other results from our laboratory suggest that hypoxia and low pH conditions actually enhance HCR of UVC-damaged DNA in SV40-transformed and primary normal human fibroblasts (Dregoesc 2007, unpublished results). The discrepancy between these latter results and findings in the current work may be due to differences in experimental procedure such as seeding efficiency, cell confluency at infection (which may affect cellular p53 activity and virus uptake, Bar et al. 2004), viral MOI in each cell line and length of exposure to oxygenated conditions during infection (i.e. reoxygenation effects). Additionally, a fewer number of determinants were used in the present analysis.

In Chapter 3 we reported that HCR of the cisplatin-damaged reporter gene was significantly reduced in SV40-transformed GM637F cells under conditions of hypoxia alone (pre- and post-infection) versus normoxic conditions when cells were scored for β-gal activity at 40 h post-infection. However, this reduction in HCR was not detectable when cells were exposed to hypoxia plus low pH conditions for only 24 h post infection and scored for β-gal activity at 24 h post-infection. These results suggest that a hypoxia treatment of greater than 24h is required for a detectable impairment of DNA repair function or that the effects of hypoxia alone are different from those of hypoxia plus low pH.

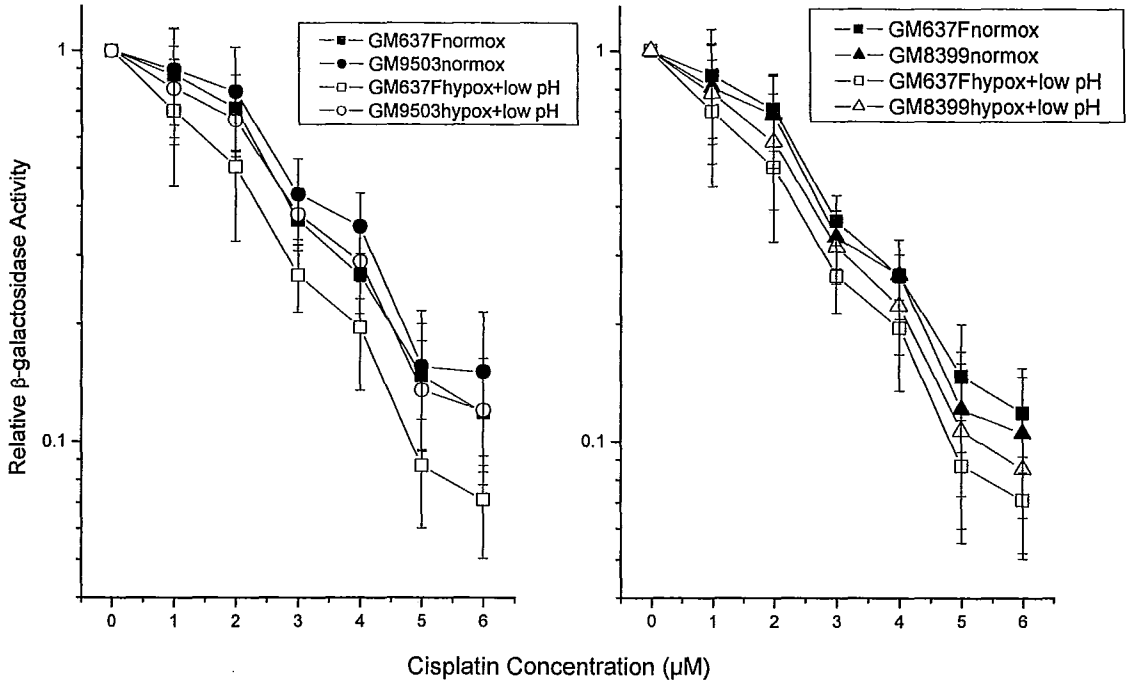

Figure 1. Left panel: HCR of β-gal activity for the cisplatin-damaged AdCA17*lacZ* virus in SV40-tansformed GM637F cells (■,□) and primary GM9503 cells (●,○) under normoxic (closed symbols) or hypoxic + low pH (open symbols) incubation conditions. Right panel: HCR in SV40-tansformed GM637F cells (■,□) and primary GM8399 cells (▲,△) under normoxic or hypoxic + low pH incubation conditions. Cells were seeded and infected 24 h later with cisplatin-treated or untreated virus for 90 min at a MOI of 80 or 140 pfu/cell. Cells were then incubated under normoxic or hypoxic + low pH (6.5) conditions for 24 h and scored for β-gal activity. Each point represents the average ± SE of 3 independent experiments each performed in triplicate.

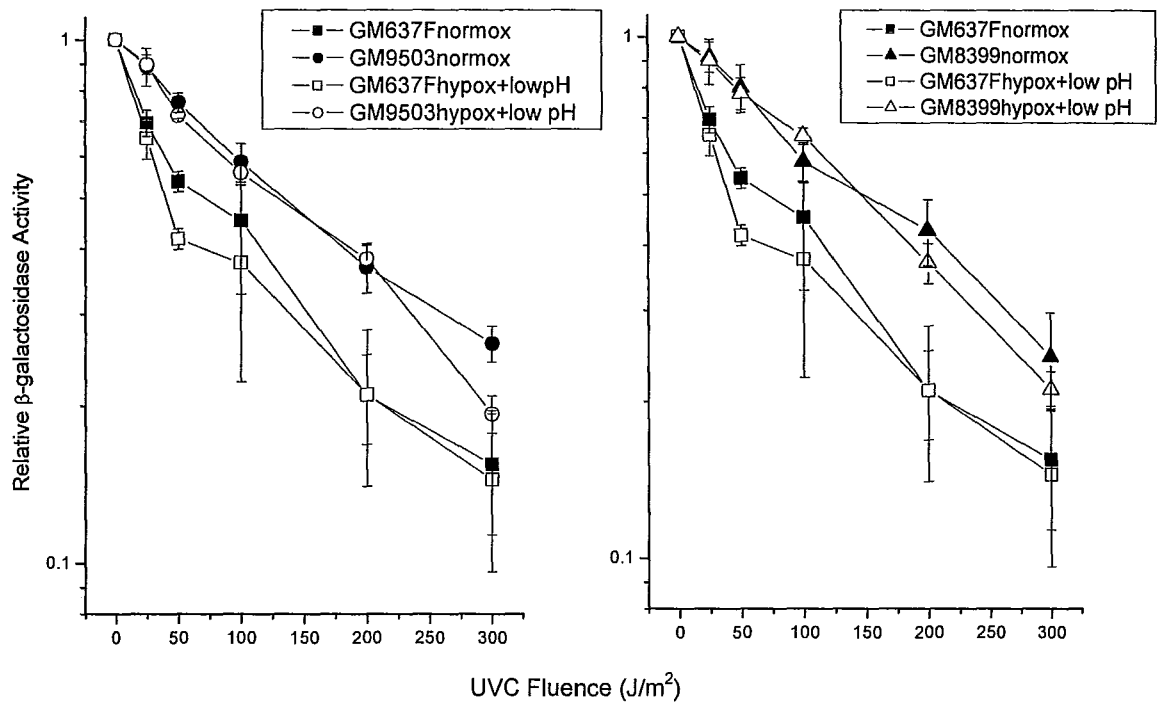

Figure 2. Left panel: HCR of β-gal activity for the UVC-damaged AdCA17*lacZ* virus in SV40-tansformed GM637F cells (■,□) and primary GM9503 cells (●,○) under normoxic (closed symbols) or hypoxic + low pH (open symbols) incubation conditions. Right panel: HCR in SV40-tansformed GM637F cells (■,□) and primary GM8399 cells (▲,△) under normoxic or hypoxic + low pH incubation conditions. Cells were seeded and infected 24 h later with UVC-treated or untreated virus for 90 min at a MOI of 80 or 140 pfu/cell. Cells were then incubated under normoxic or hypoxic + low pH (6.5) conditions for 24 h and scored for β-gal activity. Each point represents the average ± SE of 3 independent experiments each performed in triplicate.

Table I. Human normal cells used

Cell Line	Type	p53 status	Primary/Transformed
GM637F	Human NDF[a]	Abrogated p53	SV40- transformed
GM9503	Human NDF	Functional p53	Primary
GM8399	Human NDF	Functional p53	Primary

[a]Normal Diploid Fibroblast

Table II. Average D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 24 h in SV40-transformed and primary human normal fibroblasts

Cell Lines	No.	D_{37} Normoxia ± SE (μM)	D_{37} Hypoxia/low pH ± SE (μM)
GM637F (SV40)	3	3.05±0.34	2.18±0.52
GM9503 (primary)	3	3.28±0.53	3.34±0.49
GM8399 (primary)	3	3.01±0.50	2.55±0.41
Pooled primary lines	6	3.15±0.33	2.94±0.34

Table III. SV40-transformation Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 24 h in SV40-transformed versus primary human normal fibroblasts

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F/GM9503 (Normoxia)	3	0.951±0.102	0.68
GM637F/GM8399 (Normoxia)	3	1.03±0.08	0.73
GM637F/Primaries pooled (Normoxia)	6	0.991±0.061	0.89
GM637F/GM9503 (Hypoxia+low pH)	3	0.642±0.103	0.074
GM637F/GM8399 (Hypoxia+low pH)	3	0.855±0.163	0.47
GM637F/Primaries pooled (Hypoxia+low pH)	6	0.749±0.099	0.051

Table IV. Average D_{37} values from HCR of UVC-damaged AdCA17*lacZ* at 24 h in SV40-transformed and primary human normal fibroblasts

Cell Lines	No.	D_{37} Normoxia ± SE (J/m^2)	D_{37} Hypoxia/low pH ± SE (J/m^2)
GM637F (SV40)	3	114.94±27.53	100.88±44.35
GM9503 (primary)	3	204.02±23.25	199.58±11.23
GM8399 (primary)	3	226.46±29.87	200.08±14.30
Pooled primary lines	6	215.24±17.66	199.83±8.13

Table V. SV40-transformation Effects: Relative D_{37} values from HCR of UVC-damaged AdCA17*lacZ* at 24 h in SV40-transformed versus primary human normal fibroblasts

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F/GM9503 (Normoxia)	3	0.555±0.083	**0.033**[a]
GM637F/GM8399 (Normoxia)	3	0.534±0.153	0.093
GM637F/Primaries pooled (Normoxia)	6	0.544±0.078	**0.0021**[a]
GM637F/GM9503 (Hypoxia+low pH)	3	0.498±0.207	0.14
GM637F/GM8399 (Hypoxia+low pH)	3	0.488±0.189	0.11
GM637F/Primaries pooled (Hypoxia+low pH)	6	0.493±0.125	**0.0099**[a]

[a]significantly <1 by one-sample *t*-test ($P<0.05$)

Table VI. Hypoxia and Low pH Effects: Relative D_{37} values from HCR of cisplatin-damaged AdCA17*lacZ* at 24 h in SV40-transformed and primary human normal fibroblasts under hypoxic + low pH conditions versus normoxic conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F Hypoxia+low pH/Normoxia	3	0.721±0.163	0.23
GM9503 Hypoxia+low pH//Normoxia	3	1.05±0.18	0.82
GM8399 Hypoxia+low pH//Normoxia	3	0.845±0.006	**0.0014**[a]
Pooled primaries Hypoxia+low pH//Normoxia	6	0.945±0.091	0.67

[a]significantly <1 by one-sample *t*-test (P<0.05)

Table VII. Hypoxia and Low pH Effects: Relative D_{37} values from HCR of UVC-damaged AdCA17*lacZ* at 24 h in SV40-transformed and primary human normal fibroblasts under hypoxic + low pH conditions versus normoxic conditions

Cell Lines	No.	Avg. D_{37} Ratio ± SE	P Value
GM637F Hypoxia+low pH/Normoxia	3	0.804±0.157	0.34
GM9503 Hypoxia+low pH//Normoxia	3	1.01±0.15	0.96
GM8399 Hypoxia+low pH//Normoxia	3	0.903±0.093	0.41
Pooled primaries Hypoxia+low pH//Normoxia	6	0.956±0.081	0.44

CHAPTER 5

Summary and Future Directions

1.0 Summary

1.1 Conflicting Reports on the Role of MMR Proteins in Cisplatin Resistance

In the present work we sought to determine why different groups have reported conflicting findings about the role of MMR deficiency in cisplatin resistance. Our results indicate that such discrepancies may result from a failure to consider MMR effects in the context of other genetic or cellular alterations in the cell such as acquired mutations (other than MMR deficiency), immortalization-induced changes and transformation-induced changes.

In Chapter 2 we have demonstrated that while MMR deficiency may be necessary for cisplatin resistance, it may not be sufficient. Several lines of evidence from our HCR assays with MSH2, MSH6 and PMS2 knockout and wildtype MEFs all point to the conclusion that loss of any one of these proteins by itself does not predict resistance to cisplatin. Notably, we have shown that HCR of the cisplatin-damaged reporter gene is enhanced in SV40-transformed MSH2-deficient MEFs relative to proficient controls, but in contrast, we have observed no differences in repair between primary MSH2-deficient and MSH2-proficient MEFs. These results suggest that cisplatin resistance may depend upon the complex interactions of altered p53 signaling, transformation-induced changes and MMR deficiency.

In Chapter 3 we have further investigated the possible contributions of mutant p53, abrogated p53 and SV40-transformation-induced changes to repair of cisplatin-damaged DNA. We have shown in LFS cells that p53 deficiency (due to mutation of p53) does not confer a repair advantage and may not contribute to cisplatin resistance in

tumour cells. Our findings in normal human cells provide an important comparison for tumour cells and suggest that repair of cisplatin-damaged DNA under normoxic and hypoxic conditions may depend on the transformation status of the cell. In addition, we have demonstrated in p53-proficient human colon carcinoma cells that loss of hMLH1 is sufficient to confer cisplatin resistance, whereas loss of hMSH2 does not lead to a similar resistance relative to MMR-proficient controls. Taking these findings together with our previous results for murine isogenic fibroblasts (Chapter 2) further suggests that other cell alterations (aside from MMR deficiency) are required for enhanced repair of cisplatin-damaged DNA and cisplatin resistance at least in MSH2-deficient cells.

Finally, in Chapter 4 we have extended our investigation of SV40-transformation effects in normal human fibroblasts. We have found that repair of UVC-damaged DNA but not cisplatin-damaged DNA is reduced in SV40-transformed cells relative to primary cells, indicating a differential effect of abrogated p53 (due to SV40-transformation) on NER and HRR in these cells.

It is clear from our findings that loss of MMR alone may not be sufficient to confer cisplatin resistance in all cell types, underscoring the need to look at the *whole* picture when evaluating the effects of MMR status in cells. Moreover, it appears that concurrent cell alterations such as those induced by SV40-transformation (i.e. abrogated p53 and/or pRb) may contribute to enhanced repair of cisplatin-damaged DNA and cisplatin resistance in both murine and human cells.

1.2 The Use of Murine and Human Models to Study MMR Effects in Cells

While mouse knockout and wildtype cell lines provide an ideal system for evaluating MMR effects in cells, mouse models may differ in important ways to human models and the degree of evolutionary relatedness between the murine and human MMR genes may make it inappropriate to assume that the results of murine studies are directly applicable to humans. However, the use of human tumour cells presents a different set of challenges, making it more difficult to determine the precise contribution of MMR defects to cisplatin resistance in the absence of other cell alterations. Human tumour cells with MMR deficiencies are normally corrected for MMR function by chromosome transfer into cells and in some MMR-deficient cell lines, a mutant protein is expressed rather than completely absent. The effect of mutant proteins and extra genes on transferred chromosomes in these cells is currently unclear. Furthermore, human tumour cells with MMR defects are inherently mutagenic and may acquire additional mutations through continued cell passaging. These acquired mutations may have important effects in the cell that are difficult to control for and consequently, may obscure an assessment of MMR deficiency alone.

1.3 Hypoxic Effects on the Repair of DNA Damage in Cells

There is currently no consensus in the literature on the effects of hypoxia in cells. This is likely the result of inconsistencies in the conditions under which cells are examined. The severity of the hypoxic stress, length of hypoxic stress, cell types

examined and presence of concurrent adverse conditions such as low pH and DNA damage all appear to differ from study to study.

We have examined the effects of hypoxic stress (1% O_2 tension) for varying lengths of time either alone or in combination with acidosis on the repair of cisplatin- and UVC-damaged DNA in several cell types. We have shown that repair of cisplatin-damaged DNA under hypoxic conditions varies with cell type but is specifically reduced in SV40-transformed cells (Chapter 3). In murine cells (Chapter 2), we have observed both enhancements and reductions in the repair of cisplatin-damaged DNA under hypoxic conditions. In human tumour cells (Chapter 3), no enhancement effects of hypoxia on the repair of cisplatin-damaged DNA were observed although we have shown reductions in repair for both MMR-deficient and MMR-proficient tumour cells. Additionally, we have shown that hypoxia effects on repair capacity in cells may depend on the length of stress and time after infection when cells are scored for β-gal activity. Our results from Chapter 4 also indicate that the treatment of cells with low pH conditions coupled with hypoxia for 24 h post-infection is not sufficient to detect functional impairments in the repair of cisplatin- or UVC-damaged DNA in cells and detection of such differences may require a longer treatment.

Hypoxia affects multiple cellular targets in different ways, therefore it is not surprising that different cell types are differentially affected by hypoxic stress. The complex set of molecular interactions and physiological changes that take place in hypoxic cells are still under investigation and as our understanding of these hypoxia-

induced cellular changes increases, so too will our understanding of hypoxia-mediated mechanisms affecting DNA repair in mammalian cells.

1.4 In Vitro versus In Vivo Cancer Models

In the present work we have used an *in vitro* model for examining the repair of cisplatin-damaged DNA in several cell types. However, there are important differences between *in vitro* and *in vivo* conditions that are noteworthy. First, the *in vivo* tumour environment is three-dimensional (spheroid), whereas cells *in vitro* are examined in a two-dimensional monolayer. Therefore, experiments *in vitro* may not exactly mimic the types of cellular and molecular interactions that take place in the context of a three-dimensional space. Additionally, *in vitro* experiments are not able to capture the temporal and spatial fluctuations in hypoxia and variations in the internal milieu that occur *in vivo*. However, *in vitro* models have an advantage over *in vivo* models in allowing for greater control over experimental conditions, greater precision and multiple measurements to be taken simultaneously. *In vitro* models also facilitate the study of phenomenon that would be difficult to study *in vivo* such as the development of drug resistance in cells.

The *in vitro* system we have used in the present work has provided important insights into the mechanisms of cisplatin resistance in mammalian cells. Specifically, our findings have provided information on the involvement of the MMR genes, mutant p53, abrogated p53, SV40-transformation-induced changes and hypoxia in repair of cisplatin-

damaged DNA. These results present a valuable complement to studies of cisplatin resistance *in vivo*.

2.0 Future Directions

There are several aspects of the present work which could be explored further. The following experiments would provide a good addition to those presented in this thesis and would be beneficial in helping to resolve additional questions that have arisen during the course of this work:

- It would be of interest to use an MTT assay to evaluate cellular proliferation of MSH2-deficient and -proficient primary MEFs following cisplatin treatment to cells. This assay would provide an indication of the sensitivity of these cells to cisplatin and would complement the HCR data that we already have for these cells.
- An investigation of the effects of p53 deficiency and SV40-transformation in primary MSH2-deficient and MSH6-deficient MEFs (and possibly also human MMR-deficient cell lines) is warranted. An examination of these effects could be achieved with HCR experiments involving the pre-infection of cells with recombinant vectors expressing the HPV-E6 viral protein or large T antigen, both of which inactivate cellular p53. These experiments would allow us to further test whether p53 inactivation and/or SV40-transformation-induced changes contribute to cisplatin resistance in MMR-deficient cells.

- Western blots should be done to confirm whether MMR proteins such as MSH2, MSH6 and MLH1 are downregulated under hypoxic conditions in the MMR-proficient cell lines we have examined.
- It would also be of interest to examine HCR in human normal fibroblasts that have been treated with hypoxia and low pH media for 40 h post-infection as our current results indicate that detection of impairments in DNA repair may require treatment for longer than 24 h.

CHAPTER 6

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